



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C07K 14/325</b>	<b>A1</b>	(11) International Publication Number: <b>WO 99/5163</b> (43) International Publication Date: <b>14 October 1999 (14.10.99)</b>
<p>(21) International Application Number: <b>PCT/FI99/00290</b></p> <p>(22) International Filing Date: <b>6 April 1999 (06.04.99)</b></p> <p>(30) Priority Data: <b>980782 3 April 1998 (03.04.98) FI</b></p> <p>(71)(72) Applicants and Inventors: <b>KORHONEN, Timo [FI/FI]; Huntutie 10 A, FIN-00950 Helsinki (FI). PALVA, Airi [FI/FI]; Vältälöntie 87 A, FIN-00660 Helsinki (FI). PALVA, Ilkka [FI/FI]; Vältälöntie 87 A, FIN-00660 Helsinki (FI). HYNÖNEN, Ulla [FI/FI]; Raisiontie 4 A 6, FIN-00280 Helsinki (FI). WESTERLUND-WIKSTRÖM, Benita [FI/FI]; Tähtelänkuja 5 A 3, FIN-10210 Inkoo (FI).</b></p> <p>(74) Agent: <b>SEPPO LAINE OY; Itämerenkatu 3 B, FIN-00180 Helsinki (FI).</b></p>		<p>(81) Designated States: <b>AE, AL, AM, AT, AU, AZ, BA, BB, BC, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GE, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KC, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, ME, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAF patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</b></p> <p><b>Published</b> <i>With international search report.</i></p>
<p>(54) Title: <b>A PROTEIN REGION RESPONSIBLE OF BINDING TO EPITHELIAL CELL TYPES AND A DNA SEQUENCE ENCODING SAID REGION</b></p> <p>(57) Abstract</p> <p>This invention relates to a DNA molecule encoding a polypeptide responsible of binding to human and/or animal epithelial cell types. It has been found that various fragments of S-layer protein SlpA of <i>Lactobacillus brevis</i> has adhesive properties to epithelial cells types. It is possible to modify or improve the binding capacity of various prokaryotic or eucaryotic cells to human and/or animal epithelial cell types, like intestinal, urogenital and/or endothelial cell types by using lactobacillar surface structures of this invention. In particular, it is possible with the nucleotide sequences of this invention to improve the binding properties of a host cell having probiotic effects to human and/or animal epithelial cell types.</p> <p style="text-align: center;"><b>BEST AVAILABLE COPY</b></p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

## A PROTEIN REGION RESPONSIBLE OF BINDING TO EPITHELIAL CELL TYPES AND A DNA SEQUENCE ENCODING SAID REGION

The present invention relates to DNA molecules encoding polypeptides responsible of  
5 binding to human and/or animal epithelial cell types, like intestinal, urogenital and/or  
endothelial cells. Further this invention relates to vectors containing the DNA molecules and  
hosts transformed with the DNA molecules of this invention.

The present invention relates also to a method of constructing new hosts or new proteins  
10 capable of binding to human and/or animal epithelial cell types.

This invention also relates to genes encoding preselected proteins modified to bind to  
human and/or animal epithelial cell types.

15 In addition this invention relates to a host cell, to a protein and to a method for carrying  
preselected factors/properties to human and/or animal epithelial cells or cell surfaces by  
using a *Lactobacillus brevis* strain or *L. brevis* SlpA protein.

Bacterial adhesion to human epithelial and subepithelial tissues is a decisive initial event in  
20 successful colonization of tissue sites by invading bacteria. Several molecular ligand-  
receptor interactions have been characterized for bacterial species that cause infectious  
diseases in man or animals. Adhesion of pathogenic bacteria to the tissue at the infection  
site helps the bacteria to resist mechanical defences of our body, such as peristalsis in the  
intestine or flow of urine in the urinary tract. Adhesion is a key determinant in the host,  
25 tissue and cell-type tropism of bacterial infections. Attachment to tissues is also important  
for those bacteria that establish themselves as members of the normal bacterial flora in the  
human body. Species of *Lactobacillus* are major members of the indigenous bacterial flora  
in the gastrointestinal and the genital tract of man and animals. Lactobacilli are thought to  
be beneficial to their host organism and have a long history of use in the gastrointestinal and

the urogenital tract to prevent or cure various minor illnesses. Probiotic effects of lactobacilli include exclusion of invasive pathogens from intestinal and vaginal surfaces, production of antimicrobial substances, stimulation of immune systems, as well as other physiological effects. As lactobacilli are members of the normal bacterial flora and food-grade organisms, their possible use as carriers of vaccine antigens in the intestine has aroused interest. The mechanisms by which lactobacilli bring about the probiotic effects have remained uncharacterized, but it is generally agreed that efficient adhesion to epithelial surfaces is important for the colonization of the intestine as well as for the effects associated with these bacteria. Isolates of lactobacilli have been shown to adhere to the intestinal epithelium of their hosts (Coconnier *et al.*, *Appl. Environ. Microbiol* 58:2034 - 2039 (1992) and Henriksson *et al.*, *Appl. Environ. Microbiol* 57:499 - 502 (1991)), but to date, however, no molecular ligand-receptor interactions of lactobacilli have been characterized. Considering the high number of lactobacilli in our body and the biotechnological, health-associated as well as ecological importance of these bacteria, molecular characterization of their adhesion mechanisms is important.

S-layers are paracrystalline surface protein arrays that are commonly expressed by species of Eubacteria and Archaeobacteria (reviewed in Messner and Sleytr, *Adv. Microb. Physiol.* 33: 213 -275 (1992) and Sleytr and Sára, *Trends Biotechnol.* 15:20-26 (1997). Most S-layers are composed of a single protein species, the S-layer protein, greatly varying in size in different bacterial genera. The S-layer subunits are very hydrophobic and crystallize to form a two-dimensional layer on the bacterial surface. The genes encoding S-layers are efficiently transcribed, and the S-layer protein is the dominant protein species representing 10-20% of the total cellular protein of the bacterial cell. Differing functions have been attributed to the S-layers of different bacterial species. These functions include maintenance of the cell shape, protection of cells from hostile environment, anchorage of extracellular enzymes to the bacterial cell wall and mediation of bacterial attachment to animal tissues (Chu *et al.*, *J. Biol. Chem.* 266 :15258-15265 (1991), Schneitz *et al.*, *J. Appl. Microbiol.* 74: 290- 294 (1993) and Toba *et al.*, *Appl. Environ. Microbiol.* 61:2467-2471 (1995)). The S-layer of the fish pathogen *Aeromonas salmonicida* binds to extracellular matrix proteins and increases bacterial virulence by promoting bacterial spread to cause systemic infection in the

fish (Chu *et al.*, *J. Biol. Chem.* 266:15258-15265 (1991)). Most S-layer proteins aggregate in physiological buffers and their functional analysis have been restricted to solid phase assays (Sleytr and Sára, *Trends Biotechnol.* 15:20-26 (1997)), which has remained a severe limitation in the functional analysis of these important surface proteins.

5

S-layers are expressed by various species of the genus *Lactobacillus* (Masuda and Kawata *FEMS Microbiol. Lett.* 20:145-150 (1983). Their role in bacterial adhesiveness to chicken epithelium as well as to human and mouse extracellular matrix have been suggested (Schneitz *et al.*, *J. Appl. Microbiol.* 74: 290- 294 (1993), Toba *et al.*, *Appl. Environ. Microbiol.* 61:2467-2471 (1995)), but overall, the functions of lactobacillar S-layers have remained poorly characterized. Primary structure of a few lactobacillar S-layers have been determined (Vidgrén *et al.* *J. Bacteriol.* 174: 7419 - 7427 (1992), Boot *et al.*, *J. Bacteriol.* 175: 6089- 6096 (1993) and Boot *et al.*, *J. Bacteriol.* 177: 7222- 7230. The predicted lactobacillar S-layer proteins are 40 to 50 kDa in molecular size and show similarity in amino acid compositions.

15

Lactobacilli are important bacterial colonizers of our intestinal surfaces. Despite their high number and potential symbiotic effects in our body, our knowledge of the colonization mechanisms that the lactobacilli use to attach and multiply our intestine have remained uncharacterized. This has been in part due to the restricted methodology to genetically manipulate these bacteria and also due to the lack of suitable methods to study the binding mechanisms of these bacteria to intestinal or other mucosal surfaces.

20

WO 97/14802 suggests the use of *Lactobacillus fermentum* 104R 29 kD adherence factor for promoting the activity of microorganism cells to bind to a receptor recognized on mucus. However, the finding of a factor capable of binding to mucus does not solve the problem of specifically carrying preselected factors to human or animal epithelial cells. Mucus on the surface of intestinal tract and any factors bound to the mucus are easily rinsed out from the intestine.

25

In this invention a DNA molecule encoding a protein region responsible of binding of the

protein to intestinal, urogenital, endothelial and/or other epithelial cell types has for the first time been identified and characterized. Although there has been some preliminary notions about the possible binding capability of the S-layer proteins of lactic acid bacteria (LAB) to human or animal cells, the binding property has not been confirmed to be due to the S-layer protein. The DNA molecule encoding a protein region responsible of binding was  
5 unexpectedly found from a gene encoding the S-layer protein of a species of lactic acid bacteria, *Lactobacillus brevis*. However, according to this invention a homologous DNA molecule encoding similar advantageous binding properties, may be synthetic or semisynthetic or originate from the same or another group of microorganisms.

10

This invention results in various advantages. This invention makes it for the first time possible to modify or improve the binding capacity of various prokaryotic or eukaryotic cells to human or animal epithelial cell types, like intestinal, urogenital and/or endothelial cell types by using lactobacillar surface structures. In particular, it is possible with the  
15 nucleotide sequences of this invention to improve the binding properties of a host cell having probiotic effects to human and/or animal epithelial cell types.

It is possible with the nucleotide sequences of this invention to modify or improve the binding properties of a preselected protein to human and/or animal epithelial cell types.

20

Furthermore it is possible by gene technological means to modify a host cell having or being modified to have the binding capability, to carry antigens, advantageously vaccine antigens to the intestinal and/or urogenital tract of humans and/or animals. In particular, it is possible to modify strains of *Lactobacillus brevis* species.

25

The probiotic properties of various strains of *Lactobacillus brevis* species can be improved by genetic modification. Totally new properties can be transferred to the strains of *Lactobacillus brevis* species or other hosts of this invention, which may carry these properties to human or animal gastrointestinal or urogenital tract.

30

Various host cells having or being modified to have the binding capability can be used to

colonize the human or animal gastrointestinal or urogenital tract and to exclude pathogens by binding to cell receptors, which would otherwise be bound by pathogens or by growing to cell surface areas which would otherwise be colonized by pathogens.

- 5 Next, the invention will be described in more detail with the aid of the attached figures, of which

Figure 1. Effect of S-layer removal on the adherence of *L. brevis* ATCC 8287 to human Intestine 407 cells. Panel a shows adherence of untreated bacteria to the intestinal cells, and  
10 panel b shows the adhesiveness of bacteria treated with guanidine hydrochloride to remove S-layer from the bacterial surface.

Figure 2. Quantitative analysis of the effect of S-layer removal on the adherence of *L. brevis* to Intestine 407 cells. Panel A shows the number of adherent bacteria per epithelial cell; the  
15 adhesion test with the treated and the untreated bacteria was performed at four different bacterial cell densities indicated below. Panel B shows the polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) of the proteins released from the bacterial surface by the Laemmli sample buffer used in SDS-PAGE. The S-layer peptide is indicated by the arrow. The S-layer peptide is the dominant peptide species released from the cells but  
20 not the only one.

Figure 3. Schematic representation of the SlpA fragments expressed as fusion to flagellin. On top, the hydropathicity plot of the entire 465-amino acid SlpA peptide. The most probable antigenic determinant is indicated by the dashed line, and the SlpA peptide has a  
25 signal sequence of 30 amino acids. Below, the bars indicate the fragments expressed in *fliC*, the numbers refer to the N- and C-terminal amino acids in the SlpA peptide. Binding of the chimeric flagella to Intestine 407 (Int 407) cells is indicated on the right.

Figure 4. Binding of the SlpA96-200/ FliC chimeric flagella to the human Intestine 407  
30 (Panel a) and urinary bladder T24 cells (Panel c). The binding was visualized by indirect immunofluorescence. Panels b and d show the corresponding fields by light microscopy.

Panel e shows the binding of the  $\Delta$  FliC flagella to Intestine 407 cells, and Panel g the control staining without flagella; the corresponding light microscopic fields are shown in Panels f and h. Arrows indicate binding of the chimeric flagella, arrowhead indicates lack of binding.

5

Figure 5. The nucleotide sequence of *Lactobacillus brevis* SlpA gene and the corresponding amino acid sequence. The first and last nucleotide residue of the coding sequence and of the various fragments is marked with an arrow. Correspondingly the first and last amino acid residue of the entire peptide and of the fragments is marked with a circle.

10

#### DNA molecules of the invention

In this invention a DNA molecule encoding a protein region responsible of binding to human and/or animal intestinal, urogenital, endothelial and/or other epithelial cell types has for the first time been identified and characterized.

15

In this invention it has been found that the S-layer protein SlpA of *Lactobacillus brevis* has adhesive properties to epithelial cell types. The primary structure of this protein and the corresponding gene has been described in WO 94/00581 and in Vidgren *et al.*, *J. Bacteriol.* 174: 7419-7427 (1992). According to Palva, A in H. Bahl *et al.*, *FEMS Microbiology Reviews* 20 (1997):47 - 98 preliminary results have indicated that *L. brevis* S-layer protein could mediate binding to intestinal epithelial cells. However, until this date there has not been any suitable method to study the role of S-layer proteins of lactobacilli. For instance Schneitz *et al.* *J. Appl. Microbiol.* 74: 290- 294 (1993) have reported that the S-layer of *L. acidophilus* was involved in the adhesion of these bacteria to avian intestinal epithelial cells, but chemical removal of the S-layers did not affect the adhesion to Caco-2 cells. Guanidine hydrochloride extraction, which was used in the experiments, removes many protein types in addition to S-layer and hence the role of lactobacillar S-layers can not be deduced from these experiments.

25  
30

By using a recently introduced flagella display system (Westerlund-Wikström *et al.* *Protein*



*Engin. 10: 1319 - 1326 (1997))* we studied the adhesive properties of the S-layer protein SlpA of *Lactobacillus brevis*. We found, surprisingly, that the full length but also very short regions of the *Lactobacillus brevis* S-layer protein were capable of binding to epithelial cell types. The predicted size of the full-length S-layer protein is 465 amino acids. In the present invention various fragments of *slpA* were expressed as gene fusions in the variable region of the *fliC<sub>H7</sub>* gene of *Escherichia coli*. The resulting chimeric flagella were assessed by indirect immunofluorescence for binding to various epithelial cells.

The S-layer peptides needed for the binding were contained in fragments of 270, 215, 275, 150 and 105 amino acid residues respectively, the shortest fragment representing residues 96 through 200 in the S-layer protein. However, any fragment being a partial amino acid sequence of these sequences or of the entire *Lactobacillus brevis* S-layer protein and possessing similar binding capacity as the above mentioned fragments is a polypeptide of this invention and any DNA molecule encoding these polypeptides is a DNA molecule of this invention.

Chimeric flagella harbouring inserts that represented the N-terminal part of the S-layer protein bound to intestinal as well as urinary bladder cells, whereas the C-terminal part of the S-layer protein did not confer binding on the chimeric flagella. However, the C-terminal parts of the protein may have an effect in enhancing the efficiency of binding.

The S-layer expressing bacterium *Lactobacillus brevis* ATCC 8287 efficiently adhered to the human small intestinal cell line and to the human urinary bladder cell line. Bacterial adhesiveness to both cell lines was completely abolished after removal of the S-layer protein (SlpA) from the bacterial surface by guanidine hydrochloride extraction.

This invention is directed to a DNA molecule encoding a polypeptide capable of binding to human and/or animal epithelial cell types, like intestinal, urogenital and/or endothelial cells. The cells may originate from human or animal origin, like from porcine or poultry origin or from pet animals. The cells may be normal or e.g. tumour cells. The DNA molecule may be a DNA molecule having the full length or the partial sequence i.e. the coding sequence

contained in the nucleotide sequence of any one of SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5 or SEQ ID NO. 6, representing the various fragments of the *Lactobacillus brevis slpA* gene, excluding, however, the full length SEQ ID NO. 6.

5

By a partial nucleotide sequence is meant a nucleotide sequence lacking at least one nucleotide residue as compared to the nucleotide sequences of SEQ ID 1 to SEQ ID 6.

SEQ ID NO. 1 represents the 315 nucleotide residues from 286 to 600, SEQ ID NO. 2 represents the 450 nucleotide residues from 286 to 735, SEQ ID NO. 3 represents the 825 nucleotide residues from 286 to 1110, SEQ ID NO. 4 represents the 645 nucleotide residues from 91 to 735, SEQ ID NO. 5 represents the 810 nucleotide residues from 91 to 900 and SEQ ID NO. 6 represents the entire coding sequence of *Lactobacillus brevis slpA* gene from 1 to 1395 nucleotide residues. The first and last nucleotide residue of the nucleotide sequences of SEQ ID NO. 1 to SEQ ID NO. 6 is marked with an arrow in Figure 5.

The DNA molecule of this invention may be a DNA molecule encoding a polypeptide having the full length or the partial amino acid sequence i.e. the amino acid sequence contained in any one of SEQ ID NO. 7, SEQ ID NO. 8, SEQ ID NO. 9, SEQ ID NO. 10, SEQ ID NO. 11 or SEQ ID NO. 12, representing the various fragments of *Lactobacillus brevis* SlpA protein, excluding, however, the full length SEQ ID NO. 12. SEQ ID NO. 7 represents the shortest amino acid sequence of 105 amino acid residues between 96 and 200, SEQ ID NO. 8 represents 150 amino acid residues between 96 and 245, SEQ ID NO. 9 represents 275 amino acid residues between 96 and 370, SEQ ID NO. 10 represents 215 amino acid residues between 31 and 245 and SEQ ID NO. 11 represents 270 amino acid residues between 31 and 300. SEQ ID NO. 12 represents the amino acid sequence of the entire *Lactobacillus brevis* SlpA protein between 1 and 465. The first and last amino acid residue of the amino acid sequences of SEQ ID NO. 7 to SEQ ID NO. 12 is marked with a circle in Figure 5.

By a partial amino acid sequence is meant an amino acid sequence lacking at least one amino acid compared to the amino acid sequences SEQ ID NO. 7 to SEQ ID NO. 12.

The present invention furthermore relates to DNA molecules, the sequences of which  
5 differ from the sequences of the above-identified molecules due to degeneracy of the genetic code, and which code for a polypeptide capable of binding to human and/or animal epithelial cell types, like intestinal, urogenital and/or endothelial cells.

The present invention relates also to DNA molecules, the sequences of which hybridize to  
10 any one of the DNA molecules above encoding a polypeptide capable of binding to human and/or animal epithelial cell types, like intestinal, urogenital and/or endothelial cells.

The term "hybridization" in this context means hybridization under conventional hybridization conditions, preferably under stringent conditions such as described by, e.g.  
15 Sambrook *et al.* (1989, *Molecular Cloning, A Laboratory Manual* 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Typical stringent hybridization conditions are exemplified in example 7, but equal hybridizations can be carried out in slightly different conditions as is known to a person skilled in the art.

20 In example 7 the *slpA* gene of *L. brevis* has been hybridized to the chromosomal DNA of other *Lactobacillus* strains. As can be seen from example 7, the hybridization method is very useful and reliable method to find new DNA molecules of this invention. All the *L. brevis* strains tested gave positive hybridisation signal except two strains, which were shown to lack the S-layer protein. Other S-protein expressing lactobacilli belonging to other  
25 lactobacilli species gave negative result. *L. buchneri*, which is closely related to *L. brevis* gave also positive signal. *L. buchneri* carries S-layer and has the capability of binding to human and/or animal epithelial cell types, like intestinal, urogenital and/or endothelial cells.

30 These nucleic acid molecules that hybridize to the nucleic acid molecules of the present

invention can in principle be derived from any organism possessing such nucleic acid molecules. Preferably, they are derived from lactic acid bacteria or bifidobacteria. Nucleic acid molecules hybridizing to the nucleic acid molecules of the present invention can be isolated, e.g., from genomic libraries or cDNA libraries of various organisms.

5

Such nucleic acid molecules can be identified and isolated by using the nucleic acid molecules of the present invention or fragments of these molecules or the reverse complements of these molecules, e.g. by hybridization according to standard techniques (see Sambrook *et al.* (1989)).

10

As hybridization probe, e.g. nucleic acid molecules can be used that have exactly or substantially the same nucleotide sequence indicated in the Figure 5 or fragments of said sequence. Preferably is used the entire nucleotide sequence of the coding sequence of the *slpA* gene. The fragments used as hybridization probes can also be synthetic fragments obtained by conventional synthesis techniques and the sequence of which is substantially identical to that of the nucleic acid molecules according to the invention. Once genes hybridizing to the nucleic acid molecules of the invention have been identified and isolated it is necessary to determine the sequence and to analyze the properties of the proteins coded for by said sequence.

20

The term "hybridizing DNA molecule" includes fragments, derivatives and allelic variants of the above-described nucleic acid molecules that code for the above-described protein (or its equivalent) or a biologically active fragment thereof. Fragments are understood to be parts of nucleic acid molecules long enough to code for the described protein (or its equivalent) or a biologically active fragment thereof. The term "derivative" means in this context that the nucleotide sequences of these molecules differ from the sequences of the above-described nucleic acid molecules in one or more positions and are highly homologous to said sequence.

25

The present invention is directed also to DNA molecules which are homologous with the DNA molecules contained in the coding sequences of any of SEQ ID 1 to SEQ ID 6 or with

30

the DNA molecules encoding a polypeptide having the amino acid sequence contained in any of SEQ ID 7 to SEQ ID 12 or with the degenerated forms of these DNA molecules. "Homology" is understood to refer to a sequence identity of at least 50 %, preferably more than 70% and still more preferably more than 90% on the length of at least 300  
5 nucleotides. The deviations from the nucleic acid molecules described above can be the result of deletion, substitution, insertion, addition or combination.

Homology furthermore means that the respective nucleotide sequences or encoded proteins are functionally and/or structurally equivalent. The DNA molecules that are  
10 homologous to the DNA molecules described above and that are derivatives of said DNA molecules are regularly variations of said molecules which represent modifications having the same biological function. They may be naturally occurring variations, such as sequences of other organisms or mutations. These mutations may occur naturally or may be achieved by specific mutagenesis. Furthermore, these variations may be synthetically  
15 produced sequences.

The present invention furthermore relates to DNA molecules, the sequences of which have an amino acid sequence which shows at least 40 % identity, preferably at least 50 % identity, to a sequence contained above and which code for a polypeptide capable of  
20 binding to human and/or animal epithelial cell types, like intestinal, urogenital and/or endothelial cells. The amino acid sequences of this invention show identity of less than 36% compared to any known amino acid sequence. When comparing the *L. brevis* slpA protein amino acid sequence with amino acid sequences of the prior art S-layer proteins known from lactobacilli, it was found that the highest identity was below 36 %.

25 By an amino acid sequence that is an "equivalent" of a specific amino acid sequence is meant an amino acid sequence that is not identical to the specific amino acid sequence, but rather contains at least some amino acid changes (deletion, substitutions, inversions, insertions, etc.) that do not essentially affect the biological activity of the protein as compared to a  
30 similar activity of the specific amino acid sequence, when used for a desired purpose. The biological activity of a polypeptide means here the capability of binding to epithelial cells.

Preferably, an "equivalent" amino acid sequence contains at least 40% - 99% identity at the amino acid level to the specific amino acid sequence, most preferably at least 50%, more preferably at least 60% and in an especially highly preferable embodiment, at least 95% identity, at the amino acid level.

5

The term "binding" is used here to mean the adherence of a cell, protein, protein region or polypeptide reasonably firmly to an epithelial cell, like intestinal, urogenital and/ or endothelial cell type. According to this invention the binding capacity has been measured by determining the binding of polypeptides encoded by DNA molecules expressed as gene  
10 fusions in the variable region of the *fliC<sub>H7</sub>* gene of *Escherichia coli*. The resulting chimeric flagella were assessed by indirect immunofluorescence for binding to human intestinal and human urinary bladder cells. The binding capacity was visually characterized to be very strong (++++), strong (+++), weak (+) or no binding at all (-). By binding is meant the adherence of proteins or cells to the epithelial cells, particularly to the surface of the cell.

15

The binding was specific: chimeric flagella harbouring inserts that represented the N-terminal part of the S-layer protein bound to intestinal as well as urinary bladder cell types, whereas the C-terminal part of the S-layer protein did not confer binding on the chimeric flagella. The S-layer expressing bacterium *Lactobacillus brevis* ATCC 8287 efficiently  
20 adhered to the human small intestinal cell line and to the human urinary bladder cell line. Bacterial adhesiveness to both cell lines was completely abolished after removal of the S-layer protein (SlpA) from the bacterial surface by guanidine hydrochloride extraction.

In Table I is shown the differences in binding of different strains of lactic acid bacteria to  
25 human epithelial cell line (Intestine 407).

Table I. Binding of different strains of lactic acid bacteria to human epithelial cell line Intestine 407.

Strains	Binding capacity
<i>L. acidophilus</i> JCM1132	-
<i>L. crispatus</i> JCM5810	+++*
A296-21	+
<i>L. amylovorus</i> F81	-
JCM5807	+
<i>L. gallinarum</i> T-50	-
<i>L. gasseri</i> JCM1130	+
<i>L. johnsonii</i> 5F49	+
<i>L. brevis</i> ATCC 8287	++++

\* binding to the extracellular matrix secreted by the cell (see Toba *et al.*, *Appl. Environ. Microbiol.* 61:2467-2471 (1995))

As can be seen in Table I, from the lactobacilli tested only *L. brevis* binds strongly to epithelial cells, particularly to the surface of the cell.

By "lactic acid bacteria" are meant all Gram-positive; anaerobic, microaerophilic or aerotolerant; catalase negative; rods or cocci; most importantly they all produce lactic acid as sole, major or important product from the energy-yielding fermentation of sugars. In practice, genuine members of lactic acid bacteria include at least the following genera: *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Carnobacterium*, *Sporolactobacillus*, *Streptococcus*, *Enterococcus*, *Aerococcus*, *Vagococcus*, *Tetragenococcus* and *Atopium*. Many characteristics typical to genuine LAB are also common to the genus *Bifidobacterium* which consists of important health-promoting intestinal bacteria.

### Transfer of the binding property to new host cells

It is possible with the nucleotide sequences of this invention to modify or improve the binding capability of various prokaryotic or eukaryotic hosts to human and/or animal  
5 epithelial cell types, like intestinal, urogenital and/or endothelial cell types. The hosts cells are improved or modified to have the binding capability by transferring the host cells by at least one of the DNA molecules of this invention.

The binding capability may be transferred to any suitable bacterial host, for example to  
10 strains of lactic acid bacteria or bifidobacteria or to a fungal strain, like to a yeast strain.

A nucleotide sequence of this invention may be inserted into a DNA vector with conventional techniques, including blunt-ending or staggered-ending termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as  
15 appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Techniques for such manipulations are described in Sambrook *et al.* (1989, *Molecular Cloning, A Laboratory Manual* 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

20 To express a desired coding sequence, transcriptional and translational signals recognizable by the host are necessary. The nucleotide sequences of this invention may be operably linked to the transcriptional and secretory regulatory elements in an expression vector, and introduced into a host cell to produce desired protein under the control of such sequences. A DNA molecule is said to be capable of expressing a polypeptide if it contains expression  
25 control sequences which contain transcriptional regulatory information and such sequences are operably linked to the nucleotide sequence which encodes the desired polypeptide. An operable linkage is a linkage in which a sequence is connected to a regulatory sequence in such a way as to place expression of the sequence under the influence or control of the regulatory sequence.

30

Where the protein or protein region expression and secretion control sequences do not



function satisfactorily in a host cell, then sequences functional in the host cell may be substituted as necessary.

The vectors of the invention may comprise other operable linked functional elements such as DNA elements which confer antibiotic resistance on a host cell, and which provide for an origin of replication, or for insertion of a desired sequence into the chromosome of a host cell.

To transform a host cell with the DNA constructs of the invention many vector systems are available depending upon whether it is desired to insert the desired protein's DNA construct into the host cell chromosomal DNA, or to allow it to exist in an extrachromosomal form.

Cells which have stably integrated the introduced DNA into their chromosomes are selected by also introducing one or more markers which allow selection of host cells which contain the expression vector in the chromosome, for example the marker may provide resistance to antibiotics. The selectable marker gene (that can be later removed by methods well known in the art) can either be directly provided on the same vector as that providing the desired DNA gene sequences to be expressed, or such markers may be introduced into the same cell by co-transformation.

Factors of importance in selecting a particular plasmid or phage vector include the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells, which do not contain the vector and the number of copies of the vector which are desired in a particular host.

After the introduction of the vector, recipient cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the nucleotide sequences of this invention result in the production of the desired protein, or the production of a fragment of this protein and/or a host having the desired properties.

The above mentioned DNA molecules including the entire gene of *L. brevis slpA* can be

expressed on the surface of other selected well known probiotic microbes in order to enhance and target their adhesion to the host cells to allow more efficient colonization.

- Also the degree of the adhesion mediated by the polypeptides of this invention can be varied
- 5 by modifying their expression by different copy numbers, promoter strength regulation or by changing the length and amino acid composition of the binding domain with genetic engineering. With amino acid modifications encoded by the DNA molecules of this invention their host specificity may be affected if required.
- 10 Probiotics have been defined as live micro-organisms which beneficially affect the health of the host (human or animal) by improving its intestinal balance. To date, the term probiotic has been widened to include also live preparations used genitourinary to prevent infections and restore the disturbed microbiological ecological balance. Different characteristics associated with potentially health affecting bacteria may include e.g. i) acid and bile
- 15 stability, ii) adherence to intestinal cells, iii) colonization of the intestinal tract, iv) production of antimicrobial substances, v) antagonism against pathogenic bacteria and vi) safety in food and clinical use.

- According to this invention the probiotic effects of the hosts having (or modified to have)
- 20 the binding capacity to epithelial cells can be enhanced by genetic means.

- Various hosts of this invention can be used to colonize the human or animal gastrointestinal or urogenital tract and to exclude pathogens by binding to cell receptors, which would otherwise be bound by pathogens or by growing to cell surface areas, which would
- 25 otherwise be colonized by pathogens.

- As an example of establishing a surface expression of the polypeptides of this invention, one can apply well conserved cell wall binding anchors (e.g. C-terminal sequence of PrtP from *L. lactis* or other lactic acid bacteria, staphylococcal protein A, *Streptococcus*
- 30 pyogenes M6 protein, yeast anchor sequences etc.), fused to a spacer region and a DNA molecule of this invention which is preceded by a suitable promoter and signal sequence (see

e.g. Piard *et al.* *J. Bacteriol.* 179:3068-3072 (1997) and Steidler *et al.* *Appl. Environ. Microbiol.* 64: 342-345 (1998).

### Enhancing properties of oral vaccine carriers

5

The above mentioned DNA molecules including the entire gene of *L. brevis slpA* can be transferred to another microorganism which could be for example a live vaccine carrier or a new putative host developed for vaccine purpose to enhance their adhesion properties.

10 As described above the degree of adhesion can be varied by modifying the expression of the DNA molecules of this invention by different copy numbers, promoter strength regulation or by changing the length and amino acid composition of the binding domain with genetic engineering. With amino acid modifications encoded by the DNA molecules of this invention their host specificity may be affected if required.

15

The portals of entry of many pathogens are mucosal surfaces and most infections also begin at some mucosal site. The natural immune response to a pathogen is also likely to begin at the mucosal site of entry. To induce such a response with a vaccine, a similar route for its delivery may be regarded as a rational approach. Most vaccines available to date are, however, injected parenterally giving systemic response alone which may not be long lasting and protective enough. Oral vaccines are most likely to prevent intestinal diseases as they stimulate mucosal associated lymphoid tissue in the gastrointestinal tract directly. Thus, new strategies to prevent the adhesion, multiplication and invasion of pathogens at mucosal surfaces have become more acute and in some instances the only way to prevent an infectious disease. Furthermore, the use of oral (or other mucosal) routes for immunization against infective diseases is desirable because oral vaccines are easier to administer, have higher compliance rates and are likely to be less expensive. Experimental data indicate that colonization is required for example for immune response when using recombinant *Streptococcus gordonii* expressing surface antigens (Fischetti *et al.* *Current Opinion in biotechnology* 7:659-666 (1996) and Medaglini *et al.* *Proc.Natl.Acad.Sci USA* 92:6868-6872 (1995).

20  
25  
30

Mediating effective adherence to mucosal cells by the sequences of this invention, microorganisms carrying a homologous region to the identified binding region possess excellent properties as novel live oral vaccine carriers. Furthermore, it has been  
5 documented that surface (S-) layers function as effective adjuvants. In addition to the binding function, the C-terminal part of the SlpA gene can be modified to carry desired antigen epitopes and to efficiently present them as multiple copies at the S-layer formed by the SlpA subunits. Either a heterogenous or uniform S-layer can be formed depending on whether the host carries both an antigen-expressing and wild-type *slpA* gene or only the  
10 modified antigen-expressing gene. By this the amount of antigen on the cell surface may be affected. As described above, the vaccine antigen can also be expressed on the cell surface apart from a S-layer which in such a case functions as adhesion factor and adjuvant only.

Preferably expression hosts for oral vaccine carriers are strains of lactic acid bacteria and  
15 bifidobacteria, more preferably *L. brevis* species, in particular the strain *L. brevis* ATCC8287.

#### **Transfer of the binding property to new proteins**

20 In addition to the use of the polypeptides of this invention responsible of the binding property in living vaccine carriers, also non-living applications for these structures are fully possible. The desired antigen polypeptides and shorter antigen epitopes having been modified to have the binding property of this invention can be expressed in the host chosen and administered after isolation and purification as pure protein preparations. If required,  
25 such preparations may be protected against degradation by using inert particles such as biodegradable microparticles, liposomes and cochelates (O'Hagan, *Novel Delivery Systems for Oral Vaccines*. CRC Press, Florida (1994)).

### Applications of the molecules of this invention in bifunctional target-specific biomolecules

An unexplored but highly possible application for the DNA molecules of this invention are  
5 their use in bifunctional molecules i.e. by creating fusion constructs where the binding  
region mediates the adhesion to mucosal cells and the other domain functions as an active  
target-specific molecule.

Such bifunctionally acting molecules could be formed for example by combining the DNA  
10 molecules of this invention with DNA sequences encoding enzymes, single chain antibodies  
or pharmaceutical proteins or toxins.

Targeted enzymes could be applied for example in the gut for degradation of (i) lactose by  
 $\beta$ -galactosidase to decrease adverse effects in lactose intolerance or ii) milk proteins by  
15 proteases and peptidases e.g. to release bioactive peptides or to increase tolerance against  
milk allergy. Certain toxins bound specifically to the polypeptides of this invention could  
also be used for destruction of pathogens at different mucosal sites. The delivery of the  
bifunctional molecule could be by direct spray or liquid preparations (nasal mucosa), by  
melting capsules (vaginal mucosa) or by inert particles described above for vaccine delivery  
20 systems (gastrointestinal tract).

With single chain antibodies and pharmaceutical proteins linked to the binding regions of  
this invention unlimited amounts of applications for mucosal prevention and medication of  
infectious diseases can be predicted.

25

### Use of *Lactobacillus brevis* as a probiotic host

According to this invention *L. brevis* as such may function as a novel probiotic strain  
particularly due to its highly efficient binding capacity both in the gut and urinary tract even  
30 though its use in practical applications is yet unraveled. Its probiotic effect can be further  
enhanced for example by introducing genes encoding i) production of selected

bacteriocin(s) and the corresponding immunity and ii) other antimicrobial substances to antagonist pathogens and iii) enzymes increasing its metabolic activity towards available substrates to strengthen its competitiveness in the chosen niche.

- 5 Also the degree of the SlpA mediated adhesion can be varied by modifying its expression by different copy numbers, by promoter strength regulation or by changing the length and amino acid composition of the binding domain with genetic engineering. With amino acid modifications of the SlpA binding domain their host specificity may be affected if required.
- 10 In addition to the demonstrated *slpA* encoded binding capacity of *L. brevis* ATCC8287, this strain is also highly resistant in low pH and bile thus possessing the key characteristics of a probiotic strain. *L. brevis* strains have also been shown to have antagonistic effects against intestinal pathogens. Furthermore, *L. brevis* can be regularly found in the intestine of man and animals as well as in a variety of fermented food products.

15

The following examples and figures provide further details of the invention.

### Example 1

- 20 *Bacteria*-The strain ATCC 8287 of *L. brevis* and its *slpA* gene have been described (Vidgrén *et al. J. Bacteriol.* 9:7419-7427 (1992)). The bacteria were grown overnight at 37 °C in 20 ml of static MRS broth. To extract the S-layer (Masuda, K. & Kawata, T. *Microbiol. Immunol.* 23:941-953 (1979)), the bacterial cells were washed once with phosphate buffered saline, pH 7.1 (PBS) and once with distilled water, and the cells were
- 25 then suspended in 0.8 ml of distilled water. The cells were then incubated for 2 h at 37 °C with 7.2 ml of 2 M guanidine hydrochloride; the control cells were incubated similarly in PBS alone. The guanidine hydrochloride-treated cells were washed once with 2 M guanidine hydrochloride and then twice with PBS; the control cells were washed twice with PBS. For adherence assays, the bacterial cells were suspended in the cell culture medium used with
- 30 the target epithelial cell lines (see below).

*Bacterial adherence assays*-The bacterial adhesion to cultured human epithelial cells was evaluated essentially as described earlier ( Tarkkanen *et al.*, *Infect. Immunol.* 65:1546-1549 (1997)). The human small intestine Intestine 407 (ATCC CCL6) cells were cultivated to confluence in RPMI 1640 medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10 % (w/v) fetal calf serum (PAA Laboratories GmbH, Linz, Austria), 1 % (w/v) L-glutamine (Life Technologies), 1 % (w/v) nonessential amino acids (Gibco Laboratories, Grand Island, N.Y.), and gentamycin (50 µg/ml). The human urinary bladder transitional T24 cells (ATCC HTB-4) were cultered in McCoy's 5A medium (Life Technologies) supplemented with fetal calf serum, L-glutamine, and gentamycin as above. The cell lines were cultured on diagnostic glass slides (Knittel Glassbearbeitungs GmbH, Braunschweig, Germany). Before the adhesion assays, the cells were washed once with PBS. The bacteria were suspended in RPMI 1640 medium at the concentrations ranging from  $5 \times 10^7$  to  $10^9$  cells/ml, and 40 µl of the suspension per well was added to the epithelial cells and the slides were incubated for 1 h at 37 °C in a moist chamber. The slides were washed five times at room temperature with PBS for five min each and fixed for 10 min with methanol. The cells with adherent bacteria were then examined in a BX50 microscope (Olympus Optical Co., Hamburg, Germany) either directly by Nomarski interference optics (for photographing) or, for quantitative analysis, stained for 5 min with 10 % v/v Giemsa stain and analyzed by light microscopy. The number and standard deviation of adherent bacteria on 20 epithelial cells was calculated. In inhibition studies, purified Fab fragments from anti-SlpA immunoglobulins (Vidgrén *et al. J. Bacteriol.* 9:7419-7427 (1992)) or from control immunoglobulins raised against fimbrial proteins of *Escherichia coli* (Westerlund *et al. Zbl. Bakt.* 278:229-237 (1993)) were incubated with the bacterial suspensions for 30 min over crushed ice prior to the adhesion assay, the control bacteria were incubated similarly but in PBS alone. The Fab fragments were prepared by a routine procedure (Porter, *Biochem. J.* 73:119-126 (1959)) and tested at the final concentration of 500 µg/ml.

## Example 2

*Flagella display*-The principle of the flagella display system used here was recently described (Westerlund-Wikström *et al.*, *Prot. Engin.* 10:1319-1326 (1997)). Fragments

representing different parts of the *slpA* gene were amplified by polymerase chain reaction (PCR) with *Pfu* polymerase and using chromosomal DNA from the *L. brevis* strain ATCC 8287 as the template. The primers were designed on the basis of the nucleotide sequence of *slpA* (Vidgrén *et al. J. Bacteriol.* 9:7419-7427 (1992)) and contained an *AccI* restriction site at the 5' termini. The primers encoded the SlpA peptide sequences 31-245 (SEQ ID NO. 10; this peptide sequence is encoded by the nucleotide sequence SEQ ID NO. 4, which represents the nucleotide residues from 91-735), 31-300 (SEQ ID NO. 11 this peptide sequence is encoded by the nucleotide sequence SEQ ID NO. 5, which represents the nucleotide residues from 91-900), 96-200 (SEQ ID NO. 7; this peptide sequence is encoded by the nucleotide sequence SEQ ID NO. 1, which represents the nucleotide residues from 286-600), 96-245 (SEQ ID NO. 8; this peptide sequence is encoded by the nucleotide sequence SEQ ID NO. 2, which represents the nucleotide residues from 286-735), 96-370 (SEQ ID NO. 9; this peptide sequence is encoded by the nucleotide sequence SEQ ID NO. 3, which represents the nucleotide residues from 286-1110), or 239-447 (SEQ ID NO. 13; this peptide sequence is encoded by the nucleotide sequence SEQ ID NO. 14, which represents the nucleotide residues from 715-1341), where the residue numbers include the 30-mer signal sequence of the SlpA peptide. The *slpA* fragments were cloned into the *AccI* site in the plasmid pFliC<sub>HTA</sub> deleted for 174 bp in the variable region of *fliC* and expressed in trans in *E. coli* that is *fliC::Tn10* and *fimA::cat* (Westerlund-Wikström *et al Prot. Eng.* 10:1319-1326(1997)). The flagellar filaments were extracted and, after a sodium dodecylsulfate gel electrophoresis (SDS-PAGE), adjusted to an equal concentration of the FliC peptide as described recently (Westerlund-Wikström *et al., Prot. Engin.* 10:1319-1326 (1997)). The flagella lacking an insert, i.e. the  $\Delta$ FliC filaments, were available from previous work (Westerlund-Wikström *et al.*(1997)).

### Example 3

*Binding tests with chimeric flagella*-The binding of the chimeric flagella onto the epithelial cells was assessed by indirect immunofluorescence as detailed recently (Westerlund-Wikström *et al.*(1997)). Briefly, the epithelial cells were washed at room temperature with PBS, fixed with methanol for 10 min at -20 °C, and then washed with PBS at room



temperature. The flagellar extracts (40  $\mu$ l; 20  $\mu$ g/ml in PBS) were added and the slides were kept for 5 h at 4°C. After washing and a second fixing with methanol, the bound flagella were visualized by staining with immunoglobulin G molecules from an anti-H7-flagella rabbit antiserum and with fluorescein isothiocyanate-labelled secondary antibodies as detailed (Westerlund-Wikström *et al.* (1997)). The control assays included staining of the epithelial cells as above but using the  $\Delta$ FliC flagella lacking an insert, or omitting the flagellar extract, or the flagellar extract and the immunoglobulins in the staining procedure.

*Immunological methods*-For immuno electron microscopy, bacterial cells expressing the various flagellar constructs were suspended in Luria broth and immobilized on copper grids coated with Pioloform and carbon. The bacteria were left to react with an anti-H7-flagella antiserum (Westerlund-Wikström *et al.* (1997)); diluted 1/300 in PBS containing 10 mg/ml BSA or with an anti-SlpA antiserum (Vidgrén *et al. J. Bacteriol.* 9:7419-7427 (1992)); diluted 1/300 in PBS-BSA) for 90 min at room temperature. The grids were washed in PBS containing 1 mg/ml BSA, and the bound antibodies were detected with Auroprobe<sup>TM</sup>EM Protein A-conjugate (Amersham, Amersham Place; Little Chalfont, Buckinghamshire, UK; diluted 1/40). The grids were examined in a Jeol JEM-100CX transmission electron microscope at an operating voltage of 60 kV. For immunoblotting, flagellar preparations were analyzed by SDS-PAGE using a 1 % (w/v) stacking gel and a 10 % separating gel. Polypeptides were transferred onto a nitrocellulose membrane using a semi-dry transfer apparatus at 0.9 mA/cm<sup>2</sup> membrane for 2 h at 4 °C. After transfer, the membrane was quenched with PBS containing 20 mg/ml BSA for 16 h at 20 °C and washed with PBS. Polypeptides were visualized by staining with diluted polyclonal anti-flagella antibodies or anti-SlpA antibodies and alkaline-fosfatase-conjugated secondary antibodies as described (Westerlund-Wikström *et al.* (1997)). A phosphatase substrate solution containing nitroblue-tetrazolium (162  $\mu$ g/ml) and 5-bromo-4-chloro-3-indolyl-1-phosphate (370  $\mu$ g/ml) was used.

#### Example 4

*Adherence of L. brevis ATCC 8287 to intestinal cells*-We initially assessed the adhesiveness

of the *L. brevis* strain ATCC 8287 to the human small intestine cell line Intestine 407. The strain showed an efficient adhesion to the intestinal cells (Fig. 1A). The cells of *L. brevis* ATCC 8287 express the S-layer protein SlpA as their major cell surface protein (Vidgrén *et al. J. Bacteriol.* 9:7419-7427 (1992)), and we therefore extracted the S-layer from the bacterial surface and determined how this affected the adhesion. Extraction of cells with 2M guanidine hydrochloride is a routine procedure to remove bacterial S-layers, and the treatment does not lyse the bacterial cells. Treatment with guanidine hydrochloride completely abolished the adhesiveness of *L. brevis* (Fig. 1B). The S-layer peptide was the dominant peptide species released from the cells (Fig. 2B), and the results suggested a role for SlpA in bacterial adhesiveness.

### Example 5

*Expression of slpA fragments in fliC*-The *slpA* gene encoding the S-layer protein of *L. brevis* ATCC 8287 has been described (Vidgrén *et al. J. Bacteriol.* 9:7419-7427 (1992)). We cloned fragments of *slpA* into the *AccI* site in the plasmid pFliC<sub>H7Δ</sub> that contains a 174-bp deletion in the variable region of *fliC*, and the chimeric flagella were expressed in *E. coli* JT1 that is *fliC::Tn10* and *fimA::cat*. Schematic presentation of the *slpA* fragments expressed by the flagella display are shown in Fig. 3. Western blots of the flagellar preparations with anti-H7 and anti-SlpA polyclonal antibodies showed that the apparent size of the chimeric flagellins corresponded to those predicted from the nucleotide sequence, i.e. it increased with the size of the insert in  $\Delta fliC$  (data not shown). The polypeptides of smaller size that were present in the preparations and also reacted with the antibodies most likely were flagellar minor proteins (as noted earlier; Westerlund-Wikström *et al., Prot. Engin.* 10:1319-1326 (1997)) or degradation products. We noted by electron microscopy (data not shown) that the chimeric flagella expressing the larger inserts (> 200 amino acids) had short flagellar filaments and thus these preparations were reduced in the relative amount of FliC as compared to the hook and cap proteins of the flagella. The chimeric flagella SlpA31-245/ $\Delta fliC$ , SlpA31-300/ $\Delta fliC$ , SlpA96-370/ $\Delta fliC$  and SlpA239-447/ $\Delta fliC$  reacted with both the anti-H7 and the anti-SlpA antibodies, whereas the flagella with the two shortest inserts in the constructs SlpA96-245/ $\Delta fliC$  and SlpA96-200/ $\Delta fliC$  reacted with the anti-H7

antibodies and showed the expected apparent molecular size but failed to react with the anti-SlpA antiserum in Western blots.

### Example 6

5

*Binding of chimeric flagella to epithelial cells*-We next analyzed by an indirect immunofluorescence assay the binding of the chimeric flagella to Intestine 407 cells, representative examples of the assays are shown in Fig. 4. The SlpA31-300/ $\Delta$ FliC construct, carrying the N-terminal part of the molecule, exhibited binding to the Intestine 407 cells, whereas the construct SlpA239-447/ $\Delta$ FliC representing the C-terminal part, failed to bind (data not shown). No binding was observed with the  $\Delta$ FliC construct lacking an insert. These results indicated that the binding region is located within the N-terminal part of the SlpA molecule. We constructed and tested various fragments covering different regions of the N-terminus, and the shortest fragment supporting adhesion to Intestine 407 cells was the construct SlpA96-200/ $\Delta$ FliC that contained an 105 amino acid-long insert (Fig. 4A).

15

We also tested the binding of the SlpA96-200/ $\Delta$ FliC construct to the human urinary bladder cell line T24 (Fig. 4C). The chimeric flagella bound efficiently to the urinary bladder cells, whereas no binding of the  $\Delta$ FliC flagella were observed. The *L. brevis* ATCC 8287 cells exhibited an efficient adhesion to the urinary bladder cell line, and the adhesiveness was abolished after treatment of the bacteria with guanidine hydrochloride (data not shown). It was also shown that *L. brevis* ATCC 8277 cells bound to CaCo-2 cells.

20

### Example 7

25

*Hybridization of the slpA gene of L. brevis ATCC8287 to other Lactobacillus strains*-The aim of this experiment was to study the presence of *L. brevis* *slp*-gene homologs in other *Lactobacillus* strains. For this purpose chromosomal DNA was isolated from the following *L. brevis* strains: all the six strains available in the DSM culture collections (DSM 20054, 1267, 1268, 1269, 2647, 2647, 6235), five strains of the Japanese collection (Yasui *et al.* *FEMS Microbiology Letter* 133:183-186 (1995), one strain (VK3) from the culture

30

collection of TNO (delivered by prof. Peter Powels), one strain of the University of Cornell, U.S.A. (delivered by prof. Carl Batt) and one own isolate from pig intestine identified by API as *L. brevis*/*L. buchneri*. In addition, several chromosomal DNA samples of S-layer carrying *Lactobacilli* isolated from pig intestine and identified by API as putative members of *L. acidophilus*, *L. fermentum*, *L. crispatus* and *L. delbrueckii* species were tested.

Hybridization was performed in the following buffer: 5xSSC, 1% Blocking Reagent (Boehringer Mannheim), 0,02% SDS, 0,1 % lauryl sarcosine at 68°C overnight using the full length *L. brevis slpA* gene labelled with DIG (digoxigenin- dUTP, Boehringer Mannheim) as probe. Hybridization was followed by washing steps in decreasing salt concentration and increasing temperature. The final wash step was in 0.1xSSC, 0.1% SDS at 68°C for 15 min twice. The negative control used was an equal amount of *E.coli* or calf thymus DNA to that of test DNA.

15 All *L. brevis* strains tested gave a positive hybridization signal except the strain VK3 from TNO and the Japanese strain Yasui 0296961015. These two hybridization negative *L. brevis* strains were also shown to lack the S-layer protein by SDS-PAGE analysis. Furthermore, the other above mentioned S-layer expressing lactobacilli not belonging to the *L. brevis* and/or *L. buchneri* group remained negative in the hybridization test.

20 We have also shown previously in WO 94/00581 that the chromosomal DNA of *L. buchneri* strain DSM 20057 strongly hybridizes with the *slp*-gene probe used above under stringent conditions, whereas S-layer protein carrying *L. helveticus* and *L. acidophilus* strains remains hybridization negative under same conditions.

25 We have also shown that the *L. brevis*/*L. buchneri* strains hybridizing with the ATCC8287  
slp-probe also effectively bind to the Intestine 407 cells whereas non-S-layer carrying *L.*  
*brevis* strains do not adhere to Intestine 407 cells.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

## (i) APPLICANT:

- (A) NAME: Korhonen et al.  
(B) STREET: Huntutie 10 A  
(C) CITY: Helsinki  
(E) COUNTRY: Finland  
(F) POSTAL CODE (ZIP): 00950

10

- (ii) TITLE OF INVENTION: A protein region responsible of binding to  
epithelial cell types and a DNA sequence encoding said  
region

15

- (iii) NUMBER OF SEQUENCES: 16

## (iv) COMPUTER READABLE FORM:

20

- (A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

25

## (vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: FI 980782  
(B) FILING DATE: 03-APR-1998

30

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 315 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: both  
(D) TOPOLOGY: both

35

- (ii) MOLECULE TYPE: DNA (genomic)

40

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GTAAAGACCA CTAACCGTGG TTCAGTTTAC TACCGTGTTG TAACGATGGA TGGCAAGTAC 60  
CGTGGTTACG TTTATGGTGG CAAGTCTGAC ACTGCCTTTG CTGGTGGTAT CAAGTCTGCT 120  
GAAACGACTA CTAAGGCTGA TATGCCTGCA CGTACTACTG GGTTCCTACTT AACTGACACT 180  
TCAAAGAACA CTCTTTGGAC GGCTCCTAAG TACACTCAAT ACAAGGCAAG TAAAGTTAGC 240  
CTTTATGGTG TTGCTAAGGA CACCAAGTTT ACTGTAGATC AGGCTGCTAC TAAGACTCGT 300  
GAAGGTTTCAT TATAC 315

55

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 450 base pairs

28

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

5 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GTTAAGACCA CTAACCGTGG TTCAGTTTAC TACCGTGTTG TAACGATGGA TGGCAAGTAC 60  
 10 CGTGGTTACG TTTATGGTGG CAAGTCTGAC ACTGCCTTTG CTGGTGGTAT CAAGTCTGCT 120  
 GAAACGACTA CTAAGGCTGA TATGCCTGCA CGTACTACTG GGTTCCTACTT AACTGACACT 180  
 15 TCAAAGAACA CTCTTTGGAC GGCTCCTAAG TACTACTCAAT ACAAGGCAAG TAAAGTTAGC 240  
 CTTTATGGTG TTGCTAAGGA CACCAAGTTT ACTGTAGATC AGGCTGCTAC TAAGACTCGT 30  
 GAAGGTTTCAT TATACTATCA CGTAACTGCT ACTAACGGTA GTGGTATTAG TGGTTGGATT 360  
 20 TACGCTGGTA AGGGCTTCAG TACTACTGCT ACTGGTACAC AAGTACTTGG TGGTCTGTCA 420  
 ACTGATAAGT CAGTTACAGC AACCAACGAT 450

25 (2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 825 base pairs
- (B) TYPE: nucleic acid
- 30 (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GTTAAGACCA CTAACCGTGG TTCAGTTTAC TACCGTGTTG TAACGATGGA TGGCAAGTAC 60  
 CGTGGTTACG TTTATGGTGG CAAGTCTGAC ACTGCCTTTG CTGGTGGTAT CAAGTCTGCT 120  
 40 GAAACGACTA CTAAGGCTGA TATGCCTGCA CGTACTACTG GGTTCCTACTT AACTGACACT 180  
 TCAAAGAACA CTCTTTGGAC GGCTCCTAAG TACTACTCAAT ACAAGGCAAG TAAAGTTAGC 240  
 45 CTTTATGGTG TTGCTAAGGA CACCAAGTTT ACTGTAGATC AGGCTGCTAC TAAGACTCGT 300  
 GAAGGTTTCAT TATACTATCA CGTAACTGCT ACTAACGGTA GTGGTATTAG TGGTTGGATT 360  
 TACGCTGGTA AGGGCTTCAG TACTACTGCT ACTGGTACAC AAGTACTTGG TGGTCTGTCA 420  
 50 ACTGATAAGT CAGTTACAGC AACCAACGAT AACAGTGTTA AGATTGTTTA CCGTACGACT 480  
 GATGGCACTC AAGTTGGTTC TAACACTTGG GTAACCTCAA CTGATGGTAC AAAGGCAGGT 540  
 55 TCTAAGGTAA GCGATAAGGC CGCCGATCAA ACTGCTCTTG AAGCCTACAT CAATGCTAAC 600  
 AAGCCTAGCG GTTACACTGT AACTAACCTT AATGCTGCAG ATGCTACCTA TGGTAACACA 660

SUBSTITUTE SHEET (RULE 26)

29

GTTTACGCTA CTGTTTCCCA AGCAGCTACT TCTAAGGTCG CTTTGAAGGT CTCAGGGACT 720  
 CCTGTTACTA CTGCATTGAC TACAGCTGAT GCTAATGATA AGGTTGCAGC TAACGATAACC 780  
 5 ACTGCTAATG GTAGTTCTGT TGCAGGCTCA ACAGTCTATG CTGCT 825

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:  
 10 (A) LENGTH: 645 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: both  
 (D) TOPOLOGY: both

15 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

20 AAGTCATACG CTACTGCAGG TGCCTATTCA ACGTTAAAGA CGGACGCTGC TACTCGTAAC 60  
 GTCGAAGCTA CTGGTACTAA CGCTTTATAC ACGAAGCCAG GTACTGTAA GGGTGCTAAG 120  
 GTTGTGCTT CTAAGGCTAC TATGGCTAAG TTAGCTTCTT CAAAGAAGTC AGCTGACTAC 180  
 25 TTCCGTGCTT ACGGTGTAA GACCACTAAC CGTGGTTCAG TTTACTACCG TGTTGTAACG 240  
 ATGGATGGCA AGTACCGTGG TTACGTTTAT GGTGGCAAGT CTGACACTGC CTTTGCTGGT 300  
 30 GGTATCAAGT CTGCTGAAAC GACTACTAAG GCTGATATGC CTGCACGTAC TACTGGGTTC 360  
 TACTTAACTG ACACTTCAA GAACACTCTT TGGACGGCTC CTAAGTACAC TCAATACAAG 420  
 GCAAGTAAAG TTAGCCTTTA TGGTGTGCT AAGGACACCA AGTTTACTGT AGATCAGGCT 480  
 35 GCTACTAAGA CTCGTGAAGG TTCATTATAC TATCACGTAA CTGCTACTAA CGGTAGTGGT 540  
 ATTAGTGGTT GGATTTACGC TGGTAAGGGC TTCAGTACTA CTGCTACTGG TACACAAGTA 600  
 40 CTTGGTGGTC TGTCAACTGA TAAGTCAGTT ACAGCAACCA ACGAT 645

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:  
 45 (A) LENGTH: 810 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: both  
 (D) TOPOLOGY: both

50 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

55 AAGTCATACG CTACTGCAGG TGCCTATTCA ACGTTAAAGA CGGACGCTGC TACTCGTAAC 60  
 GTCGAAGCTA CTGGTACTAA CGCTTTATAC ACGAAGCCAG GTACTGTAA GGGTGCTAAG 120

SUBSTITUTE SHEET (RULE 26)

30

GTTGTCGCTT CTAAGGCTAC TATGGCTAAG TTAGCTTCTT CAAAGAAGTC AGCTGACTAC 180  
TTCCGTGCTT ACGGTGTAA GACCACTAAC CGTGGTTCAG TTTACTACCG TGTTGTAACG 240  
5 ATGGATGGCA AGTACCGTGG TTACGTTTAT GGTGGCAAGT CTGACACTGC CTTTGCTGGT 300  
GGTATCAAGT CTGCTGAAAC GACTACTAAG GCTGATATGC CTGCACGTAC TACTGGGTTT 360  
TACTTAACTG ACACTTCAAA GAACACTCTT TGGACGGCTC CTAAGTACAC TCAATACAAG 420  
10 GCAAGTAAAG TTAGCCTTTA TGGTGTGCT AAGGACACCA AGTTTACTGT AGATCAGGCT 480  
GCTACTAAGA CTCGTGAAGG TTCATTATAC TATCACGTAA CTGCTACTAA CGGTAGTGGT 540  
15 ATTAGTGGTT GGATTTACGC TGGAAGGGC TTCAGTACTA CTGCTACTGG TACACAAGTA 600  
CTTGGTGGTC TGTCAACTGA TAAGTCAGTT ACAGCAACCA ACGATAACAG TGTTAAGATT 660  
GTTTACCGTA CGACTGATGG CACTCAAGTT GGTCTAACA CTTGGGTAAC TTCAACTGAT 720  
20 GGTACAAAGG CAGGTTCTAA GGTAAGCGAT AAGGCCGCCG ATCAAAGTGC TCTTGAAGCC 780  
TACATCAATG CTAACAAGCC TAGCGGTTAC 810

25 (2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1395 base pairs  
(B) TYPE: nucleic acid  
30 (C) STRANDEDNESS: both  
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

35

(x) PUBLICATION INFORMATION:

- (A) AUTHORS: Vidgren, G  
Palva, I  
Pakkanen, R  
40 Lounatmaa, K  
Palva, A  
(B) TITLE: S-Layer Protein Gene of Lactobacillus brevis:  
Cloning by Polymerase Chain Reaction and  
Determination of the Nucleotide Sequence  
45 (C) JOURNAL: J. Bacteriol.  
(D) VOLUME: 174  
(E) ISSUE: 22  
(F) PAGES: 7419-7427  
(G) DATE: 1992  
50 (K) RELEVANT RESIDUES IN SEQ ID NO: 6: FROM 1 TO 1395

(x) PUBLICATION INFORMATION:

- (H) DOCUMENT NUMBER: WO 94/00581 A1  
(I) FILING DATE: 24-JUN-1993  
55 (J) PUBLICATION DATE: 06-JAN-1994

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

**SUBSTITUTE SHEET (RULE 26)**



31

	ATGCAATCAA	GTTTAAAGAA	ATCTCTTTAC	TTGGGCCTTG	CCGCATTGAG	CTTTGCTGGT	60
	GTTGCTGCCG	TTTCAACGAC	TGCTTCAGCT	AAGTCATACG	CTACTGCAGG	TGCCTATTCA	120
5	ACGTAAAGA	CGGACGCTGC	TACTCGTAAC	GTCGAAGCTA	CTGGTACTAA	CGCTTTATAC	180
	ACGAAGCCAG	GTAAGTTAA	GGGTGCTAAG	GTTGTCGCTT	CTAAGGCTAC	TATGGCTAAG	240
10	TTAGCTTCTT	CAAAGAAGTC	AGCTGACTAC	TTCCGTGCTT	ACGGTGTAA	GACCACTAAC	300
	CGTGGTTCAG	TTTACTACCG	TGTTGTAACG	ATGGATGGCA	AGTACCGTGG	TTACGTTTAT	360
	GGTGGCAAGT	CTGACACTGC	CTTTGCTGGT	GGTATCAAGT	CTGCTGAAAC	GACTACTAAG	420
15	GCTGATATGC	CTGCACGTAC	TACTGGGTTC	TACTTAACTG	ACACTTCAAA	GAACACTCTT	480
	TGGACGGCTC	CTAAGTACAC	TCAATACAAG	GCAAGTAAAG	TTAGCCTTTA	TGGTGTGCT	540
20	AAGGACACCA	AGTTTACTGT	AGATCAGGCT	GCTACTAAGA	CTCGTGAAGG	TTCATTATAC	600
	TATCACGTAA	CTGCTACTAA	CGGTAGTGGT	ATTAGTGGTT	GGATTTACGC	TGGTAAGGGC	660
	TTCAGTACTA	CTGCTACTGG	TACACAAGTA	CTTGGTGGTC	TGTCAACTGA	TAAGTCAGTT	720
25	ACAGCAACCA	ACGATAACAG	TGTTAAGATT	GTTTACCGTA	CGACTGATGG	CACTCAAGTT	780
	GGTTCTAACA	CTTGGGTAAC	TTCAACTGAT	GGTACAAAGG	CAGGTTCTAA	GGTAAGCGAT	840
30	AAGGCCGCCG	ATCAAAGTGC	TCTTGAAGCC	TACATCAATG	CTAACAAGCC	TAGCGGTAC	900
	ACTGTAACCTA	ACCCTAATGC	TGCAGATGCT	ACCTATGGTA	ACACAGTTTA	CGCTACTGTT	960
	TCCCAAGCAG	CTACTTCTAA	GGTCGCTTTG	AAGGTCTCAG	GGACTCCTGT	TACTACTGCA	1020
35	TTGACTACAG	CTGATGCTAA	TGATAAGGTT	GCAGCTAACG	ATACCACTGC	TAATGGTAGT	1080
	TCTGTTGCAG	GCTCAACAGT	CTATGCTGCT	GGTACTAAGT	TGGCTCAATT	AACAAGTAC	1140
40	TTGACTGGTG	AAAAGGGTCA	AGTTGTCACA	TTAACTGCCA	TCGATACTGA	TTTGGAAGAC	1200
	GCTACGTTCA	CTGGAACTAC	GACTTACTAT	TCAGATCTTG	GTAAAGCATA	CCACTACACT	1260
	TACACTTACA	ATAAGGACAG	TGCTGCTTCT	TCAAATGCAA	GTACCCAATT	TGGTTCAAAC	1320
45	GTCATGGTA	CTTTAACTGC	TACCCTTGTT	ATGGGTAAGT	CTACTGCTAC	TGCTAACGGT	1380
	ACTACTTGGT	TCAAC					1395

(2) INFORMATION FOR SEQ ID NO: 7:

50

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 105 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS:
  - (D) TOPOLOGY: linear

55

- (ii) MOLECULE TYPE: peptide

SUBSTITUTE SHEET (RULE 26)

32

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Val Lys Thr Thr Asn Arg Gly Ser Val Tyr Tyr Arg Val Val Thr Met  
 1 5 10 15  
 Asp Gly Lys Tyr Arg Gly Tyr Val Tyr Gly Gly Lys Ser Asp Thr Ala  
 20 25 30  
 Phe Ala Gly Gly Ile Lys Ser Ala Glu Thr Thr Thr Lys Ala Asp Met  
 35 40 45  
 Pro Ala Arg Thr Thr Gly Phe Tyr Leu Thr Asp Thr Ser Lys Asn Thr  
 50 55 60  
 Leu Trp Thr Ala Pro Lys Tyr Thr Gln Tyr Lys Ala Ser Lys Val Ser  
 65 70 75 80  
 Leu Tyr Gly Val Ala Lys Asp Thr Lys Phe Thr Val Asp Gln Ala Ala  
 85 90 95  
 Thr Lys Thr Arg Glu Gly Ser Leu Tyr  
 100 105

## (2) INFORMATION FOR SEQ ID NO: 8:

25

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 150 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: both

30

(ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

35

Val Lys Thr Thr Asn Arg Gly Ser Val Tyr Tyr Arg Val Val Thr Met  
 1 5 10 15  
 Asp Gly Lys Tyr Arg Gly Tyr Val Tyr Gly Gly Lys Ser Asp Thr Ala  
 20 25 30  
 Phe Ala Gly Gly Ile Lys Ser Ala Glu Thr Thr Thr Lys Ala Asp Met  
 35 40 45  
 Pro Ala Arg Thr Thr Gly Phe Tyr Leu Thr Asp Thr Ser Lys Asn Thr  
 50 55 60  
 Leu Trp Thr Ala Pro Lys Tyr Thr Gln Tyr Lys Ala Ser Lys Val Ser  
 65 70 75 80  
 Leu Tyr Gly Val Ala Lys Asp Thr Lys Phe Thr Val Asp Gln Ala Ala  
 85 90 95  
 Thr Lys Thr Arg Glu Gly Ser Leu Tyr Tyr His Val Thr Ala Thr Asn  
 100 105 110  
 Gly Ser Gly Ile Ser Gly Trp Ile Tyr Ala Gly Lys Gly Phe Ser Thr

SUBSTITUTE SHEET (RULE 26)

. 33

115 120 125

Thr Ala Thr Gly Thr Gln Val Leu Gly Gly Leu Ser Thr Asp Lys Ser  
130 135 140

5 Val Thr Ala Thr Asn Asp  
145 150

(2) INFORMATION FOR SEQ ID NO: 9:

10 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 275 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
15 (D) TOPOLOGY: both

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

20 Val Lys Thr Thr Asn Arg Gly Ser Val Tyr Tyr Arg Val Val Thr Met  
1 5 10 15

25 Asp Gly Lys Tyr Arg Gly Tyr Val Tyr Gly Gly Lys Ser Asp Thr Ala  
20 25 30

Phe Ala Gly Gly Ile Lys Ser Ala Glu Thr Thr Thr Lys Ala Asp Met  
35 40 45

30 Pro Ala Arg Thr Thr Gly Phe Tyr Leu Thr Asp Thr Ser Lys Asn Thr  
50 55 60

Leu Trp Thr Ala Pro Lys Tyr Thr Gln Tyr Lys Ala Ser Lys Val Ser  
65 70 75 80

35 Leu Tyr Gly Val Ala Lys Asp Thr Lys Phe Thr Val Asp Gln Ala Ala  
85 90 95

40 Thr Lys Thr Arg Glu Gly Ser Leu Tyr Tyr His Val Thr Ala Thr Asn  
100 105 110

Gly Ser Gly Ile Ser Gly Trp Ile Tyr Ala Gly Lys Gly Phe Ser Thr  
115 120 125

45 Thr Ala Thr Gly Thr Gln Val Leu Gly Gly Leu Ser Thr Asp Lys Ser  
130 135 140

Val Thr Ala Thr Asn Asp Asn Ser Val Lys Ile Val Tyr Arg Thr Thr  
145 150 155 160

50 Asp Gly Thr Gln Val Gly Ser Asn Thr Trp Val Thr Ser Thr Asp Gly  
165 170 175

55 Thr Lys Ala Gly Ser Lys Val Ser Asp Lys Ala Ala Asp Gln Thr Ala  
180 185 190

Leu Glu Ala Tyr Ile Asn Ala Asn Lys Pro Ser Gly Tyr Thr Val Thr

SUBSTITUTE SHEET (RULE 26)

34

195 200 205

Asn Pro Asn Ala Ala Asp Ala Thr Tyr Gly Asn Thr Val Tyr Ala Thr  
210 215 220

5 Val Ser Gln Ala Ala Thr Ser Lys Val Ala Leu Lys Val Ser Gly Thr  
225 230 235 240

10 Pro Val Thr Thr Ala Leu Thr Thr Ala Asp Ala Asn Asp Lys Val Ala  
245 250 255

Ala Asn Asp Thr Thr Ala Asn Gly Ser Ser Val Ala Gly Ser Thr Val  
260 265 270

15 Tyr Ala Ala  
275

## (2) INFORMATION FOR SEQ ID NO: 10:

- 20 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 215 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: both

25 (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

30 Lys Ser Tyr Ala Thr Ala Gly Ala Tyr Ser Thr Leu Lys Thr Asp Ala  
1 5 10 15

Ala Thr Arg Asn Val Glu Ala Thr Gly Thr Asn Ala Leu Tyr Thr Lys  
20 25 30

35 Pro Gly Thr Val Lys Gly Ala Lys Val Val Ala Ser Lys Ala Thr Met  
35 40 45

40 Ala Lys Leu Ala Ser Ser Lys Lys Ser Ala Asp Tyr Phe Arg Ala Tyr  
50 55 60

Gly Val Lys Thr Thr Asn Arg Gly Ser Val Tyr Tyr Arg Val Val Thr  
65 70 75 80

45 Met Asp Gly Lys Tyr Arg Gly Tyr Val Tyr Gly Gly Lys Ser Asp Thr  
85 90 95

Ala Phe Ala Gly Gly Ile Lys Ser Ala Glu Thr Thr Thr Lys Ala Asp  
100 105 110

50 Met Pro Ala Arg Thr Thr Gly Phe Tyr Leu Thr Asp Thr Ser Lys Asn  
115 120 125

55 Thr Leu Trp Thr Ala Pro Lys Tyr Thr Gln Tyr Lys Ala Ser Lys Val  
130 135 140

Ser Leu Tyr Gly Val Ala Lys Asp Thr Lys Phe Thr Val Asp Gln Ala

SUBSTITUTE SHEET (RULE 26)

35

```

145              150              155              160
Ala Thr Lys Thr Arg Glu Gly Ser Leu Tyr Tyr His Val Thr Ala Thr
      165              170              175
5
Asn Gly Ser Gly Ile Ser Gly Trp Ile Tyr Ala Gly Lys Gly Phe Ser
      180              185              190
10
Thr Thr Ala Thr Gly Thr Gln Val Leu Gly Gly Leu Ser Thr Asp Lys
      195              200              205
Ser Val Thr Ala Thr Asn Asp
      210              215
15 (2) INFORMATION FOR SEQ ID NO: 11:
      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 270 amino acids
            (B) TYPE: amino acid
20            (C) STRANDEDNESS:
            (D) TOPOLOGY: both
      (ii) MOLECULE TYPE: peptide
25
      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:
Lys Ser Tyr Ala Thr Ala Gly Ala Tyr Ser Thr Leu Lys Thr Asp Ala
1      5      10      15
30
Ala Thr Arg Asn Val Glu Ala Thr Gly Thr Asn Ala Leu Tyr Thr Lys
      20      25      30
35
Pro Gly Thr Val Lys Gly Ala Lys Val Val Ala Ser Lys Ala Thr Met
      35      40      45
Ala Lys Leu Ala Ser Ser Lys Lys Ser Ala Asp Tyr Phe Arg Ala Tyr
      50      55      60
40
Gly Val Lys Thr Thr Asn Arg Gly Ser Val Tyr Tyr Arg Val Val Thr
      65      70      75      80
Met Asp Gly Lys Tyr Arg Gly Tyr Val Tyr Gly Gly Lys Ser Asp Thr
      85      90      95
45
Ala Phe Ala Gly Gly Ile Lys Ser Ala Glu Thr Thr Thr Lys Ala Asp
      100      105      110
50
Met Pro Ala Arg Thr Thr Gly Phe Tyr Leu Thr Asp Thr Ser Lys Asn
      115      120      125
Thr Leu Trp Thr Ala Pro Lys Tyr Thr Gln Tyr Lys Ala Ser Lys Val
      130      135      140
55
Ser Leu Tyr Gly Val Ala Lys Asp Thr Lys Phe Thr Val Asp Gln Ala
      145      150      155      160

```

**SUBSTITUTE SHEET (RULE 26)**

	Ala	Thr	Lys	Thr	Arg	Glu	Gly	Ser	Leu	Tyr	Tyr	His	Val	Thr	Ala	Thr
						165							170			175
5	Asn	Gly	Ser	Gly	Ile	Ser	Gly	Trp	Ile	Tyr	Ala	Gly	Lys	Gly	Phe	Ser
				180					185					190		
	Thr	Thr	Ala	Thr	Gly	Thr	Gln	Val	Leu	Gly	Gly	Leu	Ser	Thr	Asp	Lys
			195					200					205			
10	Ser	Val	Thr	Ala	Thr	Asn	Asp	Asn	Ser	Val	Lys	Ile	Val	Tyr	Arg	Thr
		210					215					220				
	Thr	Asp	Gly	Thr	Gln	Val	Gly	Ser	Asn	Thr	Trp	Val	Thr	Ser	Thr	Asp
	225					230					235					240
15	Gly	Thr	Lys	Ala	Gly	Ser	Lys	Val	Ser	Asp	Lys	Ala	Ala	Asp	Gln	Thr
					245					250					255	
	Ala	Leu	Glu	Ala	Tyr	Ile	Asn	Ala	Asn	Lys	Pro	Ser	Gly	Tyr		
20				260					265					270		

(i) SEQUENCE CHARACTERISTICS:  
25 (A) LENGTH: 465 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
(D) TOPOLOGY: both

30 (ii) MOLECULE TYPE: protein

(x) PUBLICATION INFORMATION:  
(A) AUTHORS: Vidgren, G  
Palva, I  
35 Pakkanen, R  
Lounatmaa, K  
Palva, A  
(B) TITLE: S-Layer Protein Gene of Lactobacillus brevis:  
Cloning by Polymerase Chain Reaction and  
40 Determination of the Nucleotide sequence  
(C) JOURNAL: J. Bacteriol.  
(D) VOLUME: 174  
(E) ISSUE: 22  
(F) PAGES: 7419-7427  
45 (G) DATE: 1992  
(K) RELEVANT RESIDUES IN SEQ ID NO: 12: FROM 1 TO 465

(x) PUBLICATION INFORMATION:  
(H) DOCUMENT NUMBER: WO 94/00581 A1  
50 (I) FILING DATE: 24-JUN-1993  
(J) PUBLICATION DATE: 06-JAN-1994

55 Met Gln Ser Ser Leu Lys Lys Ser Leu Tyr Leu Gly Leu Ala Ala Leu  
1 5 10 15

37

	Ser	Phe	Ala	Gly	Val	Ala	Ala	Val	Ser	Thr	Thr	Ala	Ser	Ala	Lys	Ser
				20					25					30		
5	Tyr	Ala	Thr	Ala	Gly	Ala	Tyr	Ser	Thr	Leu	Lys	Thr	Asp	Ala	Ala	Thr
			35					40					45			
	Arg	Asn	Val	Glu	Ala	Thr	Gly	Thr	Asn	Ala	Leu	Tyr	Thr	Lys	Pro	Gly
		50					55					60				
10	Thr	Val	Lys	Gly	Ala	Lys	Val	Val	Ala	Ser	Lys	Ala	Thr	Met	Ala	Lys
	65					70					75				80	
	Leu	Ala	Ser	Ser	Lys	Lys	Ser	Ala	Asp	Tyr	Phe	Arg	Ala	Tyr	Gly	Val
					85					90					95	
15	Lys	Thr	Thr	Asn	Arg	Gly	Ser	Val	Tyr	Tyr	Arg	Val	Val	Thr	Met	Asp
				100					105					110		
	Gly	Lys	Tyr	Arg	Gly	Tyr	Val	Tyr	Gly	Gly	Lys	Ser	Asp	Thr	Ala	Phe
20			115					120					125			
	Ala	Gly	Gly	Ile	Lys	Ser	Ala	Glu	Thr	Thr	Thr	Lys	Ala	Asp	Met	Pro
		130					135					140				
25	Ala	Arg	Thr	Thr	Gly	Phe	Tyr	Leu	Thr	Asp	Thr	Ser	Lys	Asn	Thr	Leu
	145					150					155					160
	Trp	Thr	Ala	Pro	Lys	Tyr	Thr	Gln	Tyr	Lys	Ala	Ser	Lys	Val	Ser	Leu
				165						170					175	
30	Tyr	Gly	Val	Ala	Lys	Asp	Thr	Lys	Phe	Thr	Val	Asp	Gln	Ala	Ala	Thr
				180					185					190		
	Lys	Thr	Arg	Glu	Gly	Ser	Leu	Tyr	Tyr	His	Val	Thr	Ala	Thr	Asn	Gly
35			195					200					205			
	Ser	Gly	Ile	Ser	Gly	Trp	Ile	Tyr	Ala	Gly	Lys	Gly	Phe	Ser	Thr	Thr
		210					215					220				
40	Ala	Thr	Gly	Thr	Gln	Val	Leu	Gly	Gly	Leu	Ser	Thr	Asp	Lys	Ser	Val
	225					230					235					240
	Thr	Ala	Thr	Asn	Asp	Asn	Ser	Val	Lys	Ile	Val	Tyr	Arg	Thr	Thr	Asp
				245						250					255	
45	Gly	Thr	Gln	Val	Gly	Ser	Asn	Thr	Trp	Val	Thr	Ser	Thr	Asp	Gly	Thr
				260					265					270		
	Lys	Ala	Gly	Ser	Lys	Val	Ser	Asp	Lys	Ala	Ala	Asp	Gln	Thr	Ala	Leu
50			275					280					285			
	Glu	Ala	Tyr	Ile	Asn	Ala	Asn	Lys	Pro	Ser	Gly	Tyr	Thr	Val	Thr	Asn
		290					295					300				
55	Pro	Asn	Ala	Ala	Asp	Ala	Thr	Tyr	Gly	Asn	Thr	Val	Tyr	Ala	Thr	Val
	305					310					315					320

SUBSTITUTE SHEET (RULE 26)

[illegible]

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 209 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEO ID NO: 13:

**SUBSTITUTE SHEET (RULE 26)**



39

Thr Val Ser Gln Ala Ala Thr Ser Lys Val Ala Leu Lys Val Ser Gly  
 85 90 95  
 5 Thr Pro Val Thr Thr Ala Leu Thr Thr Ala Asp Ala Asn Asp Lys Val  
 100 105 110  
 Ala Ala Asn Asp Thr Thr Ala Asn Gly Ser Ser Val Ala Gly Ser Thr  
 115 120 125  
 10 Val Tyr Ala Ala Gly Thr Lys Leu Ala Gln Leu Thr Thr Asp Leu Thr  
 130 135 140  
 Gly Glu Lys Gly Gln Val Val Thr Leu Thr Ala Ile Asp Thr Asp Leu  
 145 150 155 160  
 15 Glu Asp Ala Thr Phe Thr Gly Thr Thr Thr Tyr Tyr Ser Asp Leu Gly  
 165 170 175  
 20 Lys Ala Tyr His Tyr Thr Tyr Thr Tyr Asn Lys Asp Ser Ala Ala Ser  
 180 185 190  
 Ser Asn Ala Ser Thr Gln Phe Gly Ser Asn Val Thr Gly Thr Leu Thr  
 195 200 205  
 25 Ala

## (2) INFORMATION FOR SEQ ID NO: 14:

- 30 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 627 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: both  
 35 (D) TOPOLOGY: both

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

40 TCAGTTACAG CAACCAACGA TAACAGTGTT AAGATTGTTT ACCGTACGAC TGATGGCACT 60  
 CAAGTTGGTT CTAACACTTG GGTAACCTCA ACTGATGGTA CAAAGGCAGG TTCTAAGGTA 120  
 AGCGATAAGG CCGCCGATCA AACTGCTCTT GAAGCCTACA TCAATGCTAA CAAGCCTAGC 180  
 45 GGTTACACTG TAACTAACCC TAATGCTGCA GATGCTACCT ATGGTAACAC AGTTTACGCT 240  
 ACTGTTTCCC AAGCAGCTAC TTCTAAGGTC GCTTTGAAGG TCTCAGGGAC TCCTGTTACT 300  
 50 ACTGCATTGA CTACAGCTGA TGCTAATGAT AAGGTTGCAG CTAACGATAC CACTGCTAAT 360  
 GGTAGTTCTG TTGCAGGCTC AACAGTCTAT GCTGCTGGTA CTAAGTTGGC TCAATTAACA 420  
 ACTGACTTGA CTGGTGAAAA GGGTCAAGTT GTCACATTAA CTGCCATCGA TACTGATTTG 480  
 55 GAAGACGCTA CGTTCCTGCTG AACTACGACT TACTATTTCAG ATCTTGGTAA AGCATACCAC 540

SUBSTITUTE SHEET (RULE 26)

40

TACACTTACA CTTACAATAA GGACAGTGCT GCTTCTTCAA ATGCAAGTAC CCAATTTGGT 600  
 TCAAACGTCA CTGGTACTTT AACTGCT 627

5 (2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1680 base pairs  
 (B) TYPE: nucleic acid  
 10 (C) STRANDEDNESS: both  
 (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

15

(ix) FEATURE:

(A) NAME/KEY: CDS  
 (B) LOCATION:189..1583

20

(ix) FEATURE:

(A) NAME/KEY: misc\_signal  
 (B) LOCATION:189..278

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

25

TCCAACGACA ATCAGAGCGT AATCCTTGTA TCTCCTTAAG GAAATCGCTA TACTTATCTT 60

CGTAGTTAGG GGATAGCTGA TCGGGTCCGC TAATGTTATG AAATAAAATT CTTAACAAAA 120

30 GCGCTAACTT CGGTTATACT ATTCTTGCTT GATAAATTAC ATATTTTATG TTTGGAGGAA 180

GAAAGATT ATG CAA TCA AGT TTA AAG AAA TCT CTT TAC TTG GGC CTT GCC 230  
 Met Gln Ser Ser Leu Lys Lys Ser Leu Tyr Leu Gly Leu Ala  
 1 5 10

35

GCA TTG AGC TTT GCT GGT GTT GCT GCC GTT TCA ACG ACT GCT TCA GCT 270  
 Ala Leu Ser Phe Ala Gly Val Ala Ala Val Ser Thr Thr Ala Ser Ala  
 15 20 25 30

40 AAG TCA TAC GCT ACT GCA GGT GCC TAT TCA ACG TTA AAG ACG GAC GCT 326

Lys Ser Tyr Ala Thr Ala Gly Ala Tyr Ser Thr Leu Lys Thr Asp Ala  
 35 40 45

GCT ACT CGT AAC GTC GAA GCT ACT GGT ACT AAC GCT TTA TAC ACG AAG 374

45 Ala Thr Arg Asn Val Glu Ala Thr Gly Thr Asn Ala Leu Tyr Thr Lys  
 50 55 60

CCA GGT ACT GTT AAG GGT GCT AAG GTT GTC GCT TCT AAG GCT ACT ATG 422

50 Pro Gly Thr Val Lys Gly Ala Lys Val Val Ala Ser Lys Ala Thr Met  
 65 70 75

GCT AAG TTA GCT TCT TCA AAG AAG TCA GCT GAC TAC TTC CGT GCT TAC 470

Ala Lys Leu Ala Ser Ser Lys Lys Ser Ala Asp Tyr Phe Arg Ala Tyr  
 80 85 90

55

GGT GTT AAG ACC ACT AAC CGT GGT TCA GTT TAC TAC CGT GTT GTA ACG 518  
 Gly Val Lys Thr Thr Asn Arg Gly Ser Val Tyr Tyr Arg Val Val Thr

**SUBSTITUTE SHEET (RULE 26)**

41

	95		100		105		110	
5	ATG GAT GGC AAG TAC CGT GGT TAC GTT TAT GGT GGC AAG TCT GAC ACT Met Asp Gly Lys Tyr Arg Gly Tyr Val Tyr Gly Gly Lys Ser Asp Thr		115		120		125	566
10	GCC TTT GCT GGT GGT ATC AAG TCT GCT GAA ACG ACT ACT AAG GCT GAT Ala Phe Ala Gly Gly Ile Lys Ser Ala Glu Thr Thr Thr Lys Ala Asp		130		135		140	614
15	ATG CCT GCA CGT ACT ACT GGG TTC TAC TTA ACT GAC ACT TCA AAG AAC Met Pro Ala Arg Thr Thr Gly Phe Tyr Leu Thr Asp Thr Ser Lys Asn		145		150		155	662
20	ACT CTT TGG ACG GCT CCT AAG TAC ACT CAA TAC AAG GCA AGT AAA GTT Thr Leu Trp Thr Ala Pro Lys Tyr Thr Gln Tyr Lys Ala Ser Lys Val		160		165		170	710
25	AGC CTT TAT GGT GTT GCT AAG GAC ACC AAG TTT ACT GTA GAT CAG GCT Ser Leu Tyr Gly Val Ala Lys Asp Thr Lys Phe Thr Val Asp Gln Ala		175		180		185	758
30	GCT ACT AAG ACT CGT GAA GGT TCA TTA TAC TAT CAC GTA ACT GCT ACT Ala Thr Lys Thr Arg Glu Gly Ser Leu Tyr Tyr His Val Thr Ala Thr		195		200		205	806
35	AAC GGT AGT GGT ATT AGT GGT TGG ATT TAC GCT GGT AAG GGC TTC AGT Asn Gly Ser Gly Ile Ser Gly Trp Ile Tyr Ala Gly Lys Gly Phe Ser		210		215		220	854
40	ACT ACT GCT ACT GGT ACA CAA GTA CTT GGT GGT CTG TCA ACT GAT AAG Thr Thr Ala Thr Gly Thr Gln Val Leu Gly Gly Leu Ser Thr Asp Lys		225		230		235	902
45	TCA GTT ACA GCA ACC AAC GAT AAC AGT GTT AAG ATT GTT TAC CGT ACG Ser Val Thr Ala Thr Asn Asp Asn Ser Val Lys Ile Val Tyr Arg Thr		240		245		250	950
50	ACT GAT GGC ACT CAA GTT GGT TCT AAC ACT TGG GTA ACT TCA ACT GAT Thr Asp Gly Thr Gln Val Gly Ser Asn Thr Trp Val Thr Ser Thr Asp		255		260		265	998
55	GGT ACA AAG GCA GGT TCT AAG GTA AGC GAT AAG GCC GCC GAT CAA ACT Gly Thr Lys Ala Gly Ser Lys Val Ser Asp Lys Ala Ala Asp Gln Thr		275		280		285	1046
60	GCT CTT GAA GCC TAC ATC AAT GCT AAC AAG CCT AGC GGT TAC ACT GTA Ala Leu Glu Ala Tyr Ile Asn Ala Asn Lys Pro Ser Gly Tyr Thr Val		290		295		300	1094
65	ACT AAC CCT AAT GCT GCA GAT GCT ACC TAT GGT AAC ACA GTT TAC GCT Thr Asn Pro Asn Ala Ala Asp Ala Thr Tyr Gly Asn Thr Val Tyr Ala		305		310		315	1142
70	ACT GTT TCC CAA GCA GCT ACT TCT AAG GTC GCT TTG AAG GTC TCA GGG Thr Val Ser Gln Ala Ala Thr Ser Lys Val Ala Leu Lys Val Ser Gly		320		325		330	1190

SUBSTITUTE SHEET (RULE 26)

42

ACT CCT GTT ACT ACT GCA TTG ACT ACA GCT GAT GCT AAT GAT AAG GTT 1238  
 Thr Pro Val Thr Thr Ala Leu Thr Thr Ala Asp Ala Asn Asp Lys Val  
 335 340 345 350

5 GCA GCT AAC GAT ACC ACT GCT AAT GGT AGT TCT GTT GCA GGC TCA ACA 1286  
 Ala Ala Asn Asp Thr Thr Ala Asn Gly Ser Ser Val Ala Gly Ser Thr  
 355 360 365

10 GTC TAT GCT GCT GGT ACT AAG TTG GCT CAA TTA ACA ACT GAC TTG ACT 1334  
 Val Tyr Ala Ala Gly Thr Lys Leu Ala Gln Leu Thr Thr Asp Leu Thr  
 370 375 380

15 GGT GAA AAG GGT CAA GTT GTC ACA TTA ACT GCC ATC GAT ACT GAT TTG 1382  
 Gly Glu Lys Gly Gln Val Val Thr Leu Thr Ala Ile Asp Thr Asp Leu  
 385 390 395

20 GAA GAC GCT ACG TTC ACT GGA ACT ACG ACT TAC TAT TCA GAT CTT GGT 143  
 Glu Asp Ala Thr Phe Thr Gly Thr Thr Thr Tyr Tyr Ser Asp Leu Gly  
 400 405 410

AAA GCA TAC CAC TAC ACT TAC ACT TAC AAT AAG GAC AGT GCT GCT TCT 1478  
 Lys Ala Tyr His Tyr Thr Tyr Thr Tyr Asn Lys Asp Ser Ala Ala Ser  
 415 420 425 430

25 TCA AAT GCA AGT ACC CAA TTT GGT TCA AAC GTC ACT GGT ACT TTA ACT 1526  
 Ser Asn Ala Ser Thr Gln Phe Gly Ser Asn Val Thr Gly Thr Leu Thr  
 435 440 445

30 GCT ACC CTT GTT ATG GGT AAG TCT ACT GCT ACT GCT AAC GGT ACT ACT 1574  
 Ala Thr Leu Val Met Gly Lys Ser Thr Ala Thr Ala Asn Gly Thr Thr  
 450 455 460

35 TGG TTC AAC TAATAATTAT TATTTAGGTG AGCTTTGTTG ATAAAAAGGT 1623  
 Trp Phe Asn  
 465

CTTTTCACG TTTATGTTGG GGAGACCTTT TTATATTGAA AAAATTAGGC CTTTGTT 168

40 (2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 465 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

50 Met Gln Ser Ser Leu Lys Lys Ser Leu Tyr Leu Gly Leu Ala Ala Leu  
 1 5 10 15

55 Ser Phe Ala Gly Val Ala Ala Val Ser Thr Thr Ala Ser Ala Lys Ser  
 20 25 30

Tyr Ala Thr Ala Gly Ala Tyr Ser Thr Leu Lys Thr Asp Ala Ala Thr

SUBSTITUTE SHEET (RULE 26)

43

	35		40		45
	Arg Asn Val Glu Ala Thr Gly Thr Asn Ala Leu Tyr Thr Lys Pro Gly				
	50		55		60
5	Thr Val Lys Gly Ala Lys Val Val Ala Ser Lys Ala Thr Met Ala Lys				
	65		70		75
	Leu Ala Ser Ser Lys Lys Ser Ala Asp Tyr Phe Arg Ala Tyr Gly Val				
10		85		90	95
	Lys Thr Thr Asn Arg Gly Ser Val Tyr Tyr Arg Val Val Thr Met Asp				
		100		105	110
15	Gly Lys Tyr Arg Gly Tyr Val Tyr Gly Gly Lys Ser Asp Thr Ala Phe				
		115		120	125
	Ala Gly Gly Ile Lys Ser Ala Glu Thr Thr Thr Lys Ala Asp Met Pro				
20		130		135	140
	Ala Arg Thr Thr Gly Phe Tyr Leu Thr Asp Thr Ser Lys Asn Thr Leu				
	145		150		155
	Trp Thr Ala Pro Lys Tyr Thr Gln Tyr Lys Ala Ser Lys Val Ser Leu				
25		165		170	175
	Tyr Gly Val Ala Lys Asp Thr Lys Phe Thr Val Asp Gln Ala Ala Thr				
		180		185	190
30	Lys Thr Arg Glu Gly Ser Leu Tyr Tyr His Val Thr Ala Thr Asn Gly				
		195		200	205
	Ser Gly Ile Ser Gly Trp Ile Tyr Ala Gly Lys Gly Phe Ser Thr Thr				
		210		215	220
35	Ala Thr Gly Thr Gln Val Leu Gly Gly Leu Ser Thr Asp Lys Ser Val				
		225		230	235
	Thr Ala Thr Asn Asp Asn Ser Val Lys Ile Val Tyr Arg Thr Thr Asp				
40		245		250	255
	Gly Thr Gln Val Gly Ser Asn Thr Trp Val Thr Ser Thr Asp Gly Thr				
		260		265	270
45	Lys Ala Gly Ser Lys Val Ser Asp Lys Ala Ala Asp Gln Thr Ala Leu				
		275		280	285
	Glu Ala Tyr Ile Asn Ala Asn Lys Pro Ser Gly Tyr Thr Val Thr Asn				
		290		295	300
50	Pro Asn Ala Ala Asp Ala Thr Tyr Gly Asn Thr Val Tyr Ala Thr Val				
		305		310	315
	Ser Gln Ala Ala Thr Ser Lys Val Ala Leu Lys Val Ser Gly Thr Pro				
55		325		330	335
	Val Thr Thr Ala Leu Thr Thr Ala Asp Ala Asn Asp Lys Val Ala Ala				

SUBSTITUTE SHEET (RULE 26)

340

345

350

Asn Asp Thr Thr Ala Asn Gly Ser Ser Val Ala Gly Ser Thr Val Tyr  
355 360 365

5

Ala Ala Gly Thr Lys Leu Ala Gln Leu Thr Thr Asp Leu Thr Gly Glu  
370 375 380

	Lys	Gly	Gln	Val	Val	Thr	Leu	Thr	Ala	Ile	Asp	Thr	Asp	Leu	Glu	Asp
10	385					390					395					400

Ala Thr Phe Thr Gly Thr Thr Thr Tyr Tyr Ser Asp Leu Gly Lys Ala  
405 410 415

15 Tyr His Tyr Thr Tyr Thr Tyr Asn Lys-Asp Ser Ala Ala Ser Ser Asn  
420 425 430

Ala Ser Thr Gln Phe Gly Ser Asn Val Thr Gly Thr Leu Thr Ala Thr  
435 440 445

20

Leu Val Met Gly Lys Ser Thr Ala Thr Ala Asn Gly Thr Thr Trp Phe  
450 455 460

	Asn
25	465

What we claim is:

1. A DNA molecule encoding a polypeptide capable of binding to human and/or animal  
5 epithelial cell types, said DNA molecule being selected from the group consisting of:
  - (a) DNA molecules having at least the partial coding sequences of any one of SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO 5 and/or SEQ ID NO. 6 excluding the full length SEQ ID NO 6;
  - 10 (b) DNA molecules encoding a polypeptide having at least the partial amino acid sequences of any one of SEQ ID NO. 7, SEQ ID NO. 8, SEQ ID NO.9, SEQ ID NO. 10, SEQ ID NO. 11 and/or SEQ ID NO 12, excluding the full length SEQ ID NO 12;
  - (c) DNA molecules the coding sequences of which differ from the coding sequence of a nucleic acid molecule of (a) or (b) due to the degeneracy of the genetic code;
  - 15 (d) DNA molecules hybridizing under stringent conditions to a molecule of (a), (b) and/or (c) excluding the full-length SEQ ID NO 6 ; and
  - (e) DNA molecules encoding a polypeptide capable of binding to human and/or animal epithelial cell types and having an amino acid sequence which shows at least 40 % identity to a sequence contained in (b) excluding the full length SEQ ID NO 12.
- 20 2. The DNA molecule of claim 1 encoding a polypeptide capable of binding to human and/or animal epithelial cell types, wherein said polypeptide is capable of binding to intestinal, urogenital and/or endothelial cells.
- 25 3. The DNA molecule of claim 1 or 2, which originates from the DNA molecule encoding *Lactobacillus brevis* S-layer SlpA protein.
4. A vector containing a DNA molecule of any one of claim 1, 2 or 3.
- 30 5. The vector of claim 4, in which the DNA molecule is operably linked to expression and optionally to secretion control sequences allowing expression in prokaryotic or eukaryotic

host cells.

6. A host cell transformed with a DNA molecule of any one of claims 1, 2 or 3 or with a vector of claim 4 or 5.

5

7. A method of constructing a host cell capable of binding to human and/or animal epithelial cell types, like intestinal, urogenital and/or endothelial cell types, comprising transforming the cell with at least one DNA molecule selected from the group consisting of:

- 10 (a) DNA molecules having at least the partial coding sequences of any one of SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5, and/or SEQ ID NO. 6;
- (b) DNA molecules encoding a polypeptide having at least the partial amino acid sequences of any one of SEQ ID NO. 7, SEQ ID NO. 8, SEQ ID NO. 9, SEQ ID NO. 10, SEQ ID NO. 11 and/or SEQ ID NO. 12 ;
- 15 (c) DNA molecules, the coding sequence of which differ from the coding sequence of a DNA molecule of (a) or (b) due to the degeneracy of the genetic code;
- (d) DNA molecules hybridizing under stringent conditions to a molecule of (a), (b) and/or (c); and
- (e) DNA molecules encoding a polypeptide capable of binding to human and/or animal
- 20 epithelial cell types, like intestinal, urogenital and/or endothelial cell types and having an amino acid sequence which shows at least 40 % identity to a sequence contained in (b).

8. A host cell constructed by the method of claim 7.

25 9. The host cell of any one of claims 6 or 8, which has probiotic effects.

10. The host cell of claim 9, wherein the probiotic effects have been enhanced by genetic means.

30 11. The host cell of claim 9 or 10, which belongs to lactic acid bacteria or bifidobacteria.

12. The host cell of any one of claims 6 or 8 to 11, wherein the host cell is a vaccine carrier.



13. The host cell of any one of claims 6 or 8 to 12, wherein the host cell has been genetically modified to carry at least one of the factors selected from the group consisting of an antigen, an epitope, a single chain antibody, an enzyme, a pharmaceutical protein and a toxin.

5

14. A method of constructing a polypeptide capable of binding to human and/or animal epithelial cell types, like intestinal, urogenital and/or endothelial cell types, comprising modifying the gene encoding the polypeptide with a DNA molecule of claim 1 or 7.

10

15. A gene encoding a preselected protein, wherein the gene encoding the protein is genetically modified to bind to human and/or animal epithelial cell types, like intestinal, urogenital and/or endothelial cell types with at least one DNA molecule selected from the group consisting of any of the sequences of claim 1 or 7.

15

16. The gene of claim 15, wherein the preselected protein is selected from the group consisting of an antigen, an epitope, a single chain antibody, an enzyme, a pharmaceutical protein and a toxin.

17. A polypeptide encoded by a DNA molecule of claim 1 or 15.

20

18. A *Lactobacillus brevis* strain for carrying preselected factors/ properties to human and/or animal epithelial cells or cell surfaces, wherein the strain has the capability of binding to human and/or animal epithelial cell types and wherein the gene encoding the S-layer SlpA protein is genetically modified to carry at least one of the factors/properties selected from the group consisting of an antigen, an epitope, a single chain antibody, an enzyme, a pharmaceutical protein and a toxin.

25

19. A *Lactobacillus brevis* strain for carrying preselected factors/ properties to human and/or animal epithelial cells or cell surfaces, wherein the strain has the capability of binding to human and/or animal epithelial cell types and wherein the strain is genetically modified to have improved probiotic effects.

30

20. A *Lactobacillus brevis* S-layer SlpA protein for carrying preselected factors/ properties to human and/or animal epithelial cells or cell surfaces, wherein the protein has the capability of binding to human and/or animal epithelial cell types and wherein the gene encoding the S-layer SlpA protein is genetically modified to carry at least one of the
- 5 factors/properties selected from the group consisting of an antigen, an epitope, a single chain antibody, an enzyme, a pharmaceutical protein and a toxin.
21. A method for carrying preselected factors/ properties to human and/or animal epithelial cells or cell surfaces by using a *Lactobacillus brevis* strain, wherein the strain has the
- 10 capability of binding to human and/or animal epithelial cell types and wherein the gene encoding the S-layer SlpA protein is genetically modified to carry at least one of the factors/properties selected from the group consisting of an antigen, an epitope, a single chain antibody, an enzyme, a pharmaceutical protein and a toxin.
- 15 22. A method for carrying preselected factors/ properties to human and/or animal epithelial cells or cell surfaces by using a *Lactobacillus brevis* strain, wherein the strain has the capability of binding to human and/or animal epithelial cell types and wherein the strain is genetically modified to have improved probiotic effects.
- 20 23. A method for carrying preselected factors/ properties to human and/or animal epithelial cells or cell surfaces by using the *Lactobacillus brevis* S-layer SlpA protein, wherein the protein has the capability of binding to human and/or animal epithelial cell types and wherein the gene encoding the S-layer SlpA protein is genetically modified to carry at least
- 25 one of the factors/properties selected from the group consisting of an antigen, an epitope, a single chain antibody, an enzyme, a pharmaceutical protein and a toxin.
24. The use of the host cells of any one of claims 6 or 8 to 13 for excluding pathogens in the cell surfaces of the gastrointestinal or urogenital tract of humans and/ or animals.

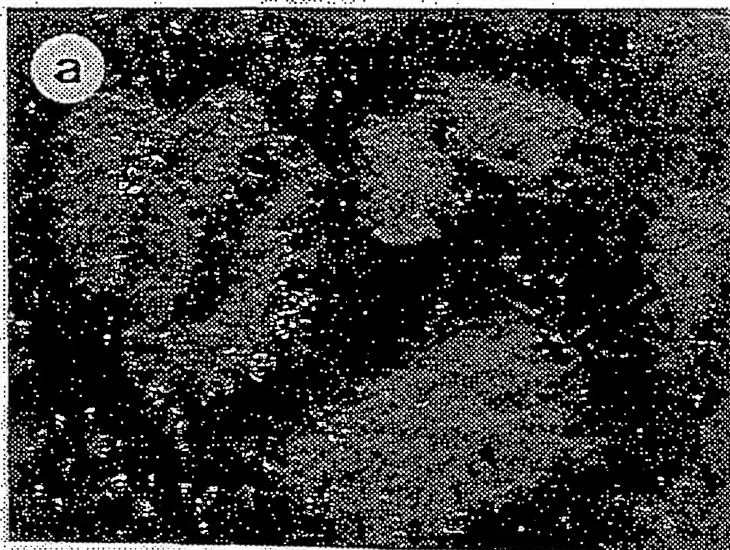


Fig. 1A



Fig. 1B

2 / 8

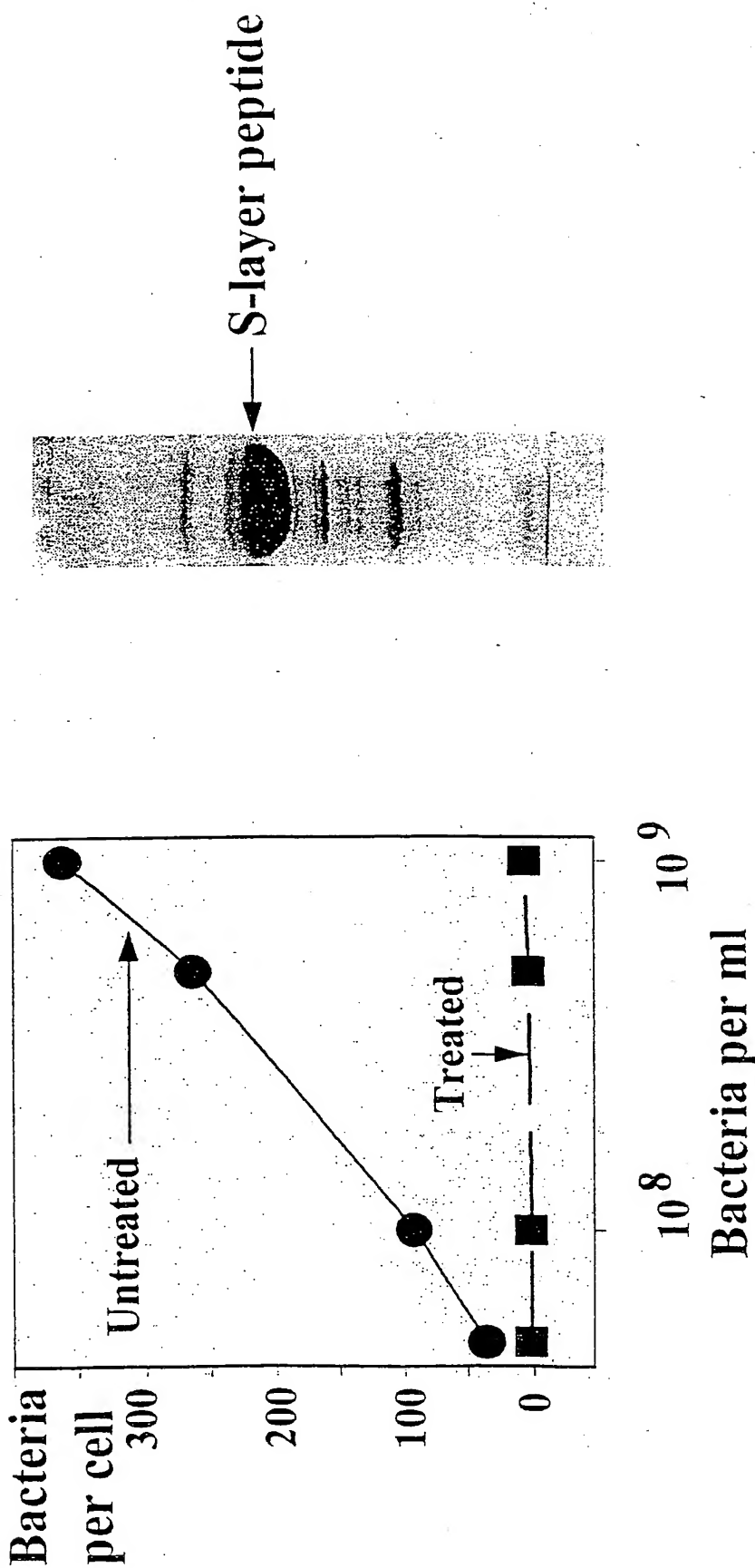


Fig. 2B

Fig. 2A

3/8

# Expression of *slpA* fragments as fusions to *fliC*

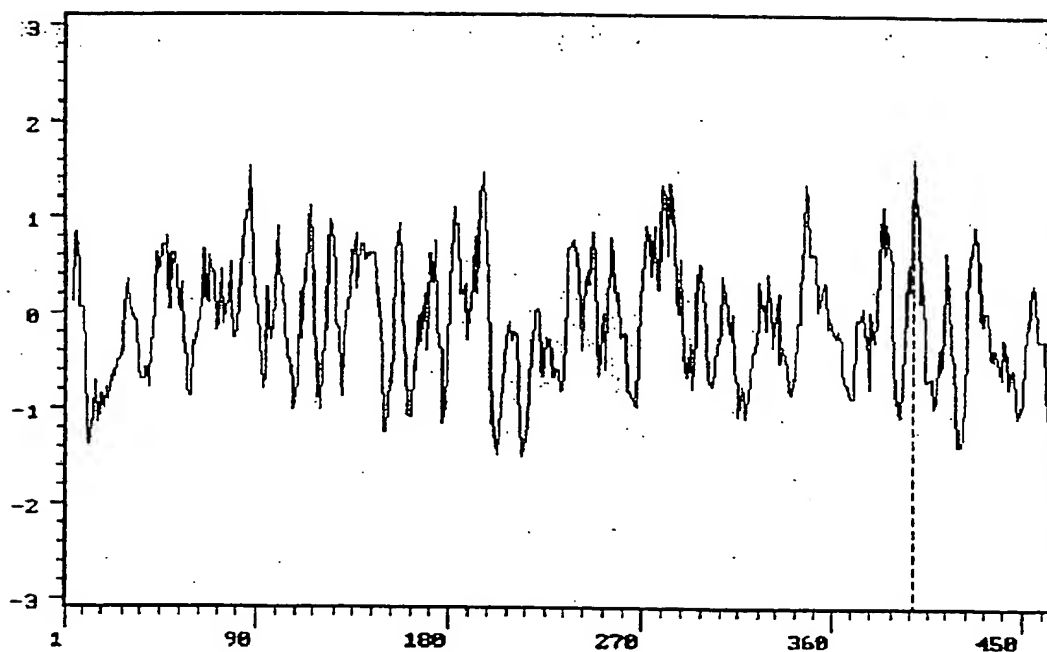


Fig. 3A

Binding to  
Int 407 cells

31		300	+
31		245	+
96		370	+
96		245	+
96		200	+
239		447	-

Fig. 3B

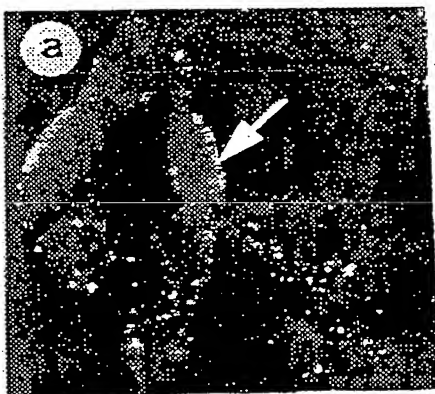


Fig. 4A

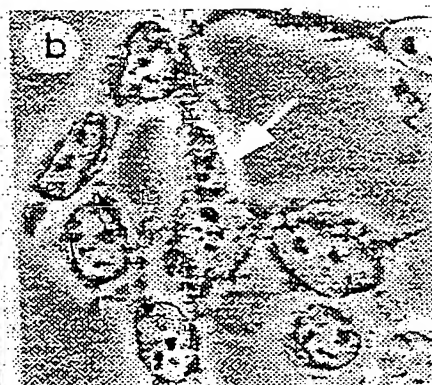


Fig. 4B

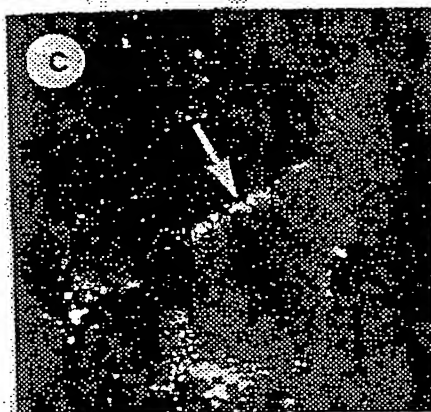


Fig. 4C

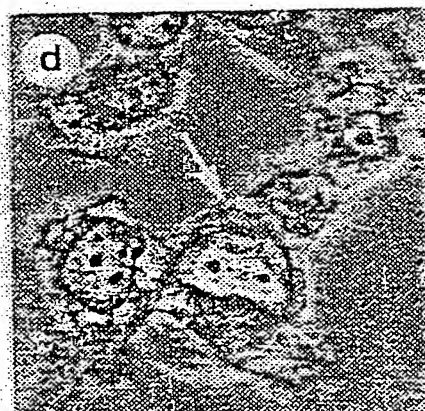


Fig. 4D



Fig. 4E



Fig. 4F

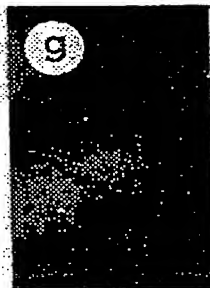


Fig. 4G



Fig. 4H

5/8

TCCAACGACAATCAGAGCGTAATCCTTGTATCTCCTTAAGGAAATCGCTATACTTATCTT

CGTAGTTAGGGGATAGCTGATCGGGTCCGCTAATGTTATGAAATAAAATTCTTAACAAAA

GCGCTAACTTCGGTTATACTATTCTTGCTTGATAAATTACATATTTTATGTTTGGAGGAA

1  
↓  
GAAAGATTATGCAATCAAGTTTAAAGAAATCTCTTTACTTGGGCCTTGCCGCATTGAGCT  
(M) Q S S L K K S L Y L G L A A L S  
( slpA coding region  
1

91  
↓  
TTGCTGGTGTGCTGCCGTTTCAACGACTGCTTCAGCTAAGTCATACGCTACTGCAGGTG  
F A G V A A V S T T A S A (K) S Y A T A G  
31

CCTATTCAACGTTAAAGACGGACGCTGCTACTCGTAACGTCGAAGCTACTGGTACTAACG  
A Y S T L K T D A A T R N V E A T G T NCTTTATACACGAAGCCAGGTACTGTTAAGGGTGCTAAGGTTGTCGCTTCTAAGGCTACTA  
A L Y T K P G T V K G A K V V A S K A T

286  
↓  
TGGCTAAGTTAGCTTCTTCAAAGAAGTCAGCTGACTACTTCCGTGCTTACGGTGTTAAGA  
M A K L A S S K K S A D Y F R A Y G (V) K  
96

Fig. 5A

6/8

CCACTAACCGTGGTTCAGTTTACTACCGTGGTGTAAACGATGGATGGCAAGTACCGTGGTT  
T T N R G S V Y Y R V V T M D G K Y R G

ACGTTTATGGTGGCAAGTCTGACACTGCCTTTGCTGGTGGTATCAAGTCTGCTGAAACGA  
Y V Y G G K S D T A F A G G I K S A E T

CTACTAAGGCTGATATGCCTGCACGTACTACTGGGTTCTACTTAACTGACACTTCAAAGA  
T T K A D M P A R T T G F Y L T D T S K

ACACTCTTTGGACGGCTCCTAAGTACACTCAATACAAGGCAAGTAAAGTTAGCCTTTATG  
N T L W T A P K Y T Q Y K A S K V S L Y

GTGTTGCTAAGGACACCAAGTTTACTGTAGATCAGGCTGCTACTAAGACTCGTGAAGGTT  
G V A K D T K F T V D Q A A T K T R E G

600  
↓  
CATTATACTATCACGTAAGTCTGCTACTAACGGTAGTGGTATTAGTGGTTGGATTTACGCTG  
S L (Y) Y H V T A T N G S G I S G W I Y A  
200

GTAAGGGCTTCAGTACTACTGCTACTGGTACACAAGTACTTGGTGGTCTGTCAACTGATA  
G K G F S T T A T G T Q V L G G L S T D

715                      735  
↓                      ↓  
AGTCAGTTACAGCAACCAACGATAACAGTGTTAAGATTGTTTACCGTACGACTGATGGCA  
K (S) V T A T N (D) N S V K I V Y R T T D G  
239                      245

Fig. 5B



7/8

CTCAAGTTGGTTCTAACACTTGGGTAACCTCAACTGATGGTACAAAGGCAGGTTCTAAGG  
T Q V G S N T W V T S T D G T K A G S K

TAAGCGATAAGGCCGCGGATCAAACCTGCTCTTGAAGCCTACATCAATGCTAACAAGCCTA  
V S D K A A D Q T A L E A Y I N A N K P

900  
↓  
GCGGTTACACTGTAACCTAACCCTAATGCTGCAGATGCTACCTATGGTAACACAGTTTACG  
S G (Y) T V T N P N A A D A T Y G N T V Y  
300

CTACTGTTTCCCAAGCAGCTACTTCTAAGGTCGCTTTGAAGGTCTCAGGGACTCCTGTTA  
A T V S Q A A T S K V A L K V S G T P V

CTACTGCATTGACTACAGCTGATGCTAATGATAAGGTTGCAGCTAACGATACCACTGCTA  
T T A L T T A D A N D K V A A N D T T A

1110  
↓  
ATGGTAGTTCTGTTGCAGGCTCAACAGTCTATGCTGCTGGTACTAAGTTGGCTCAATTAA  
N G S S V A G S T V Y A (A) G T K L A Q L  
370

CAACTGACTTGACTGGTGAAAAGGGTCAAGTTGTACATTAACTGCCATCGATACTGATT  
T T D L T G E K G Q V V T L T A I D T D

TGGAAGACGCTACGTTCACTGGAACTACGACTTACTATTTCAGATCTTGGTAAAGCATACC  
L E D A T F T G T T T Y Y S D L G K A Y

Fig. 5C

8/8

ACTACACTTACACTTACAATAAGGACAGTGCTGCTTCTTCAAATGCAAGTACCCAATTTG  
H Y T Y T Y N K D S A A S S N A S T Q F

1341  
↓  
GTTCAAACGTCACCTGGTACTTTAACTGCTACCCTTGTTATGGGTAACTCTACTGCTACTG  
G S N V T G T L T (A) T L V M G K S T A T  
447

1395  
↓  
CTAACGGTACTACTTGGTTCAACTAATAATTATTATTTAGGTGAGCTTTGTTGATAAAAA  
A N G T T W F (N)  
465

GGTCTTTTCAACGTTTATGTTGGGGAGACCTTTTTATATTGAAAAAATTAGGCCTTTGTT

Fig. 5D

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 99/00290

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
IPC6: C07K 14/325 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols)		
IPC6: C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
SE,DK,FI,NO classes as above		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
WPI, PATENT ABSTRACTS OF JAPAN, STRAND, CASEARCH, BIOSIS, MEDLINE, SCISEARCH, LIFE SCIENCES COLLECTION		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FEMS Microbiology Reviews, Volume 20, 1997, Hubert Bahl et al, "IV. Molecular biology of S-layers", page 47 - page 98, see pages 82-83	18-23
A	--	1-17,24
A	WO 9400581 A1 (VIAGEN OY), 6 January 1994 (06.01.94)	1-24
A	Journal of Bacteriology, Volume 174, No 22, 1992, Gabriele vidgrén et al, "S-Layer Protein Gene of Lactobacillus brevis: Cloning by Polymerase ChainReaction and Determinaiton of the Nucleotide Sequence" page 7419 - page 7427	1-24
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
26 July 1999		28-07-1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86		Authorized officer  Hampus Rystedt/Eö Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 99/00290

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>Journal of Applied Bacteriology, Volume 74, 1993, C. Schneitz et al, "Adhesion of Lactobacillus acidophilus to avian intestinal epithelial cells mediated by the crystalline bacterial cell surface layer (S-layer)" page 290 - page 294</p> <p style="text-align: center;">--</p>	1-24
A	<p>Chemical Abstracts, Volume 126, No 13, 31 March 1997 (31.03.97), (Columbus, Ohio, USA), Savijoki, Kirsi et al, "High level heterologous protein production in Lactococcus and Lactobacillus using a new secretion system based on the Lactobacillus brevis S-layer signals", page 1, THE ABSTRACT No 167032, Gene 1997, 186 (2), 255-262</p> <p style="text-align: center;">-- -----</p>	1-24

# INTERNATIONAL SEARCH REPORT

Information on patent family members

01/07/99

International application No.

PCT/FI 99/00290

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9400581 A1	06/01/94	AU 4329793 A	24/01/94

Form PCT/ISA/210 (patent family annex) (July 1992)

**THIS PAGE BLANK (USPTO)**

Fig. 1A



Fig. 1B



2/8

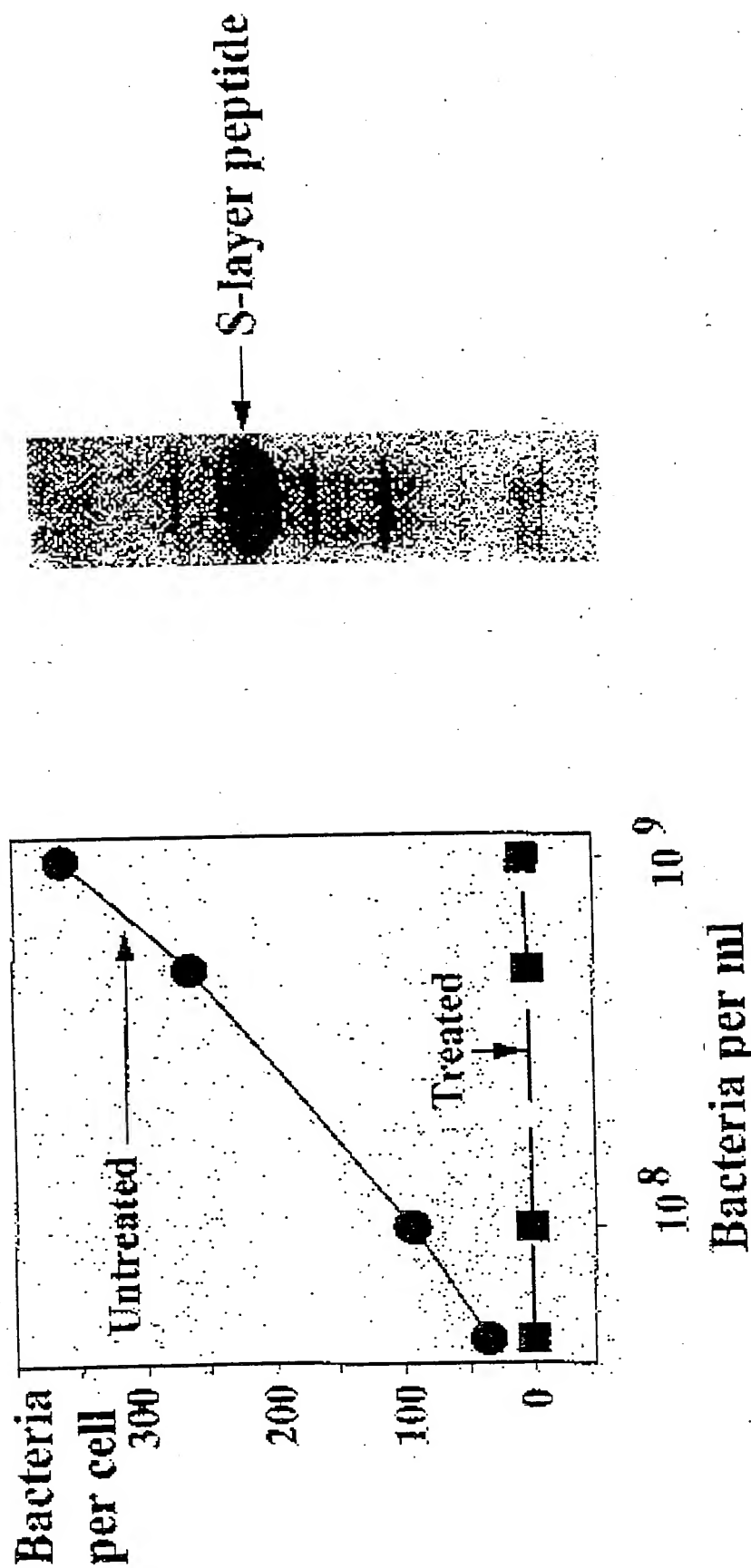


Fig. 2B

Fig. 2A



# Expression of *slpA* fragments as fusions to *fliC*

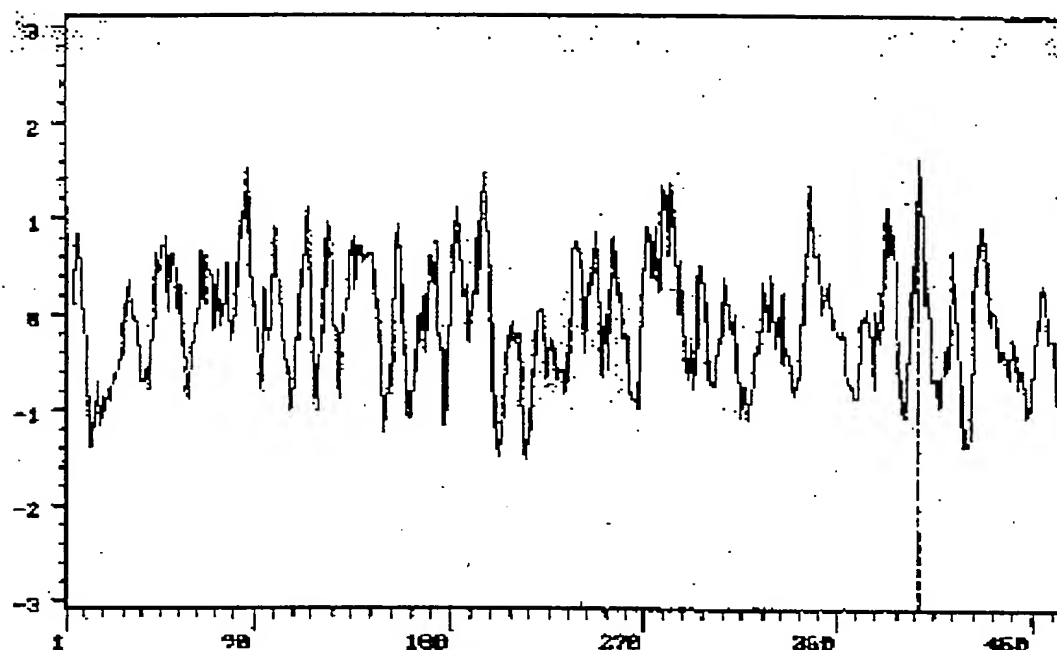


Fig. 3A

Binding to  
Int 407 cells

31		300	+
31		245	+
96		370	+
96		245	+
96		200	+
239		447	-

Fig. 3B



Fig. 4A



Fig. 4B

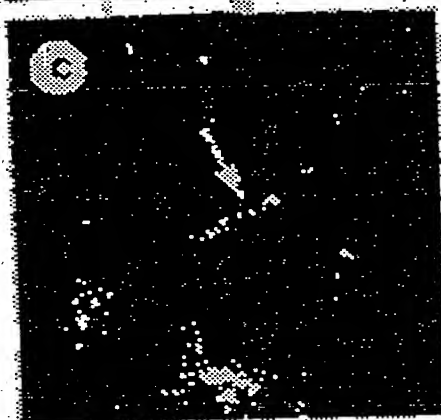


Fig. 4C



Fig. 4D



Fig. 4E



Fig. 4F

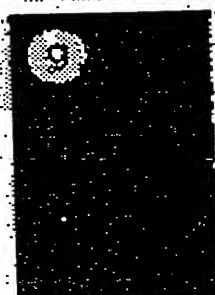


Fig. 4G



Fig. 4H

5/8

TCCACGGACAAATCAGAGCGTAATCCTTGTATCTCTCTTACGGAATCGCTATACTTATCTT

CCTAATTAGGGGATAGCTGATCGGCTCGCTAATGTTATGAAATAAAATTCTTACAGAA

GCGCTAAGCTTCGGTTATACTATTCTTCTCTTGTATATTAATATATTTATGTTTGAGGGA

GAAGATTATGCAATCAGTTTAAAGAAATCTCTTTACTTGGGGCTTGGGGCATTGAGCT

(M) Q S S L K K S L Y L G L R A L S  
( alpha coding region  
1

TTGCTGGTGTGCTGCGTTTCAACCGACTGCTTCAGCTAAGTCATACGCTACTGCAGGTG  
F A G V A A V S T T A S A (K) S Y A T A G  
31

CCTATTCAACGTTAAGACGGACGCTGCTACTCCTAACGTCGAAGCTACTGGTACTAAGC  
A Y S T L K T D A A T R N V E A T G T H

CTTTATACACGAAGCCAGGTAAGTATTAAAGGCTGCTAAGCTTGTGCTTCTAAGGCTACTA  
A L Y T K P G T V K G A K V V A S K A T

TGGCTAAGTTAGCTTCTTCAAGAAAGTCAGCTGACTACTTCCGTGCTTAAGGTGTTAAGA  
M A K L A S S K K S A D Y F R A Y G (V) K  
96

Fig. 5A

6/8

CCACTAACCGTGGTTTCAGTTTACTACCGTGGTTGTAACGATGGATGGCAAGTACCGTGGTT  
T T N R G S V Y Y R V V T M D G K Y R G

ACGTTTATGGTGGCAAGTCTGACACTGCLTTTGGCTGGTATCAAGTCTGGTGAACCA  
Y V Y G G K S D T A F A G G I K S A E T

CTACTAACGGTGAATATGCGTGGTACTACTGGGTTCTACTTAACTGACAUTTCWAGA  
T T K A D M P A R T T G F Y L T D T S K

ACACTCTTTGGACGGCTGCTAAGTACACTCAATACAAGCCAGTAAAGTTAGCTTTATC  
N T L W T A P K Y T Q Y K A S K V S L Y

GTGTTGCTAAGGACACCAAGTTTACTGTAGATCAGGCTGCTACTAAGACTCGTGAGGTT  
G V A K D T K F T V D Q A A T K T R E G

600  
↓  
CATTATCTATCAGCTAAGTCTACTAAGGTAAGTGGTATTAAGTGGTTGGATTTACGGCTG  
S L (Y) Y H V T A T N G S G I S G W I Y A  
200

GTAAGGGCTTCACTACTACTGCTACTGCTACACAGTACTTGGTGGTCTCTCAACTGATA  
G K C P S T T A T G T Q V L G G L S T D

715 735  
↓ ↓  
AGTCACTTACAGCAACCAACGATAACCACTGTTAAGATTCTTTACCGTACGACTGATGGCA  
K (S) V T A T N (D) H S V K I V Y R T T D G  
239 245

Fig. 5B

7/8

CTCAAGTTGGTTCTAACACTTGGGTAAGTTCACTGATGGTACAAAGGCAGTTCTAAGG  
T Q V G S N T W V F S T D G T K A G S K

TAAGCGATAAGGCGCGCGATCAAACTGCTCTTGAAGGCTACATCAATGCTAACAAAGGCTA  
V S D K A A D Q T A I E A Y I N A N K F

900  
↓  
GGGTTAAGCTCTAAGCTAACCTTAATGCTGCGAGTCTACCTATGCTAACACAGTTTACG  
S G (Y) T V T N P N A A D A T Y G N T V Y  
300

CTACTGTTTTCCAGGCAGCTACTTCTAAGGTGCGTTTGAAGGTCTCAGGGACTCCTGTTA  
A T V S Q A A T S K V A I K V S G T P V

CTACTGCAATTGACTACAGCTGCTGCTAATGATAAGGTTCCAGCTAACGATACCACTGCTA  
T T A L T T A D A N D K V A A N D T T A

1110  
↓  
ATGCTAGTTCTGTTGGAGGCTCAACAGTCTATGCTGCTGGTACTTAAGTTGGCTCAATTAA  
N S S S V A G S T V Y A (A) G T K L A Q L  
370

CAAGTCACTTGAAGTGGTGAAGGCTCAAGTTGTCACATTAACTGGCATCGATACTGATT  
T T D L T G E K G Q V V T L T A I D T D

TGAAGAGCGCTACCTTCACTGGAAGTACGACTTACTATTCAAGATCTTGGTAAAGCATACG  
L E D A T F T C T T T Y Y S D I G K A Y

Fig. 5C

8/8

ACTACACTTACACTTACAAATAAGGACAGTGCCTGCCTTCCTTCAATGCAAGTACCCCATTTG  
 H Y T Y T Y N K D S A A S S N A S T Q F

1341  
 GTTCAGACGTCACTGGTACTTTTAACTGCTACCCCTTGTATTCGGTAAGTCTACTGCTACTG  
 G S N V T G T L T (A) T L V M G K S T A T  
 447

1395  
 CTAACGGTACTACTTGGTTCACCTAATAATTATATTAGGTGAGCTTTCTTGATATAAA  
 A N G T T W E (N)  
 455

GGTCTTTTCAACGTTTATGTTGGGGAGACCTTTTATATTTGAATAAATTAGGCCTTTGTT

Fig. 5D

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**

**THIS PAGE BLANK (USPTO)**